



eCOMMONS

Loyola University Chicago
Loyola eCommons

Master's Theses

Theses and Dissertations

1980

Studies on the Mechanism of Reaction of Papain with N-Acetyl-L-Phenylalanyl Glycinal, a Model for an Elementary Step in Catalysis

Thresiamma Kuppy
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_theses



Part of the [Biochemistry, Biophysics, and Structural Biology Commons](#)

Recommended Citation

Kuppy, Thresiamma, "Studies on the Mechanism of Reaction of Papain with N-Acetyl-L-Phenylalanyl Glycinal, a Model for an Elementary Step in Catalysis" (1980). *Master's Theses*. 3180.
https://ecommons.luc.edu/luc_theses/3180

This Thesis is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Master's Theses by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.



This work is licensed under a [Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License](#).
Copyright © 1980 Thresiamma Kuppy

STUDIES ON THE MECHANISM OF REACTION OF PAPAIN
WITH N-ACETYL-L-PHENYLALANYL
GLYCINAL, A MODEL
FOR AN ELEMENTARY STEP
IN CATALYSIS

by

Thresiamma Kuppy

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Master of Science
December
1980

DEDICATION

This thesis is dedicated to my parents and my husband, Simon.

ACKNOWLEDGMENTS

I would like to acknowledge my gratitude to Dr. Allen Frankfater for all his assistance and guidance in my studies.

Also, I wish to thank the Faculty and Staff of the Department of Biochemistry and Biophysics for the financial assistance and cooperation.

VITA

Thresiamma was born to Joseph and Aliekutty Tharakan in Shertallay, India, on November 4, 1953. She attended St. Mary's High School, Shertallay, India, graduated with honors in 1969, and was awarded a National Merit Scholarship for higher education. Following graduation from high school, she entered St. Teresa's College of Kerala University majoring in Chemistry.

On December 31, 1972, she was married to Simon Joseph Kuppy. Following her marriage, she moved to the United States and continued her undergraduate studies at Aurora College in Aurora, Illinois. She graduated in 1976 with honors.

Ms. Kuppy continued her graduate education in the Department of Biochemistry and Biophysics, Loyola University, under Dr. Allen Frankfater, Associate Professor of Biochemistry, in July 1976.

Ms. Kuppy has one daughter, JoAnna.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
VITA	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
Chapter	
I. INTRODUCTION	1
X-ray Studies	2
Reaction of Papain with Substrates	4
Evidence for an Acyl Enzyme Intermediate	5
Role of the Individual Amino Acids at the Active Site	8
Cysteine 25	8
Aspartic Acid 158	8
Histidine 159	9
Asparagine 175	10
Tryptophan 177	10
Evidence for the pK's of the Active Site Residues.	11
Alternative Mechanisms for Papain Catalysis	12
Acylation	15
Deacylation	15
Reaction of Papain with Aldehydes ,	16
II. MATERIALS.	19
Synthesis of N-acetyl-L-phenylalanyl glycinal	19
Synthesis of Organomercurial Sepharose ,	21
Affinity Purification of Papain	21
Preparation of Affinity Column	21
Determination of Column Capacity	22
Papain Purification	22
III. METHODS	23
Measurement of K_I for N-acetyl-L-phenylalanyl glycinal	23
Enzyme Assay.	24
Fluorescence Studies.	24
Measurement of Dissociation of Papain Aldehyde Complex.	25

	Page
Measurement of pH Dependency of the Fluorescence of papain and Aldehyde bound papain.	25
IV. KINETIC DERIVATIONS	26
V. RESULTS	32
Preparation of N-acetyl-L-phenylalanyl glycinal.	32
Dissociation Constants for N-acetyl-L-phenyl- alanyl glycinal with Papain	32
Fluorescence of Affinity purified Papain and its N-acetyl-L-phenylalanyl glycinal Complex.	38
Measurement of the Rate of Dissociation of Papain N-acetyl-L-phenylalanyl glycinal complex	41
Second Order Rate constants for the Association of Papain with N-acetyl-L-phenylalanyl glycinal.	47
VI. DISCUSSION.	51
Introduction	51
pH Dependency of Equilibrium Association Constant for N-acetyl-L-phenylalanyl glycinal with Papain	55
pH Dependency of the Dissociation Rate Constant.	57
pH Dependency of Second Order Rate Constant for the Association of Aldehyde to Papain	57
Ionization of Amino Acid Residues at the Papain Active Site.	58
Effects of the Hydration of the Aldehyde on the Kinetic Constants	60
Identity of the Active Site Nucleophile.	61
REFERENCES.	63

LIST OF TABLES

		Page
Table I	Limiting values of kinetic constants and corresponding pK values for papain catalyzed hydrolysis of p-nitrophenyl, benzyl and methyl esters of Z lysine	6
Table II	pH dependency of the equilibrium dissociation constant for N-acetyl-L-phenylalanyl glycinal with papain	36
Table III	pH dependency of the individual kinetic constants for the association of N-acetyl-L-phenylalanyl glycinal with papain	49
Table IV	Limiting values for kinetic parameters of the Association of N-acetyl-L-phenylalanyl glycinal with papain	50

LIST OF FIGURES

	Page
Figure 1 - Inhibition of papain catalyzed hydrolysis of CGN by N-acetyl-L-phenylalanyl glycinal	34
Figure 2 - Determination of the equilibrium dissociation constant for N-acetyl-L-phenylalanyl glycinal with papain at pH 5.0	35
Figure 3 - The pH Dependency of the equilibrium association constant for N-acetyl-L-phenylalanyl glycinal with papain	37
Figure 4 - Fluorescence Emission Spectra of papain in the presence and absence of N-acetyl-L-phenylalanyl glycinal at pH 6.5	39
Figure 5 - pH dependency of fluorescence emission of papain at 344nm in the presence and absence of N-acetyl-L-phenylalanyl glycinal	40
Figure 6 - Determination of the pseudofirst order rate constants for the dissociation of the N-acetyl-L-phenylalanyl glycinal papain complex in the presence of varying concentrations of tetrathionate	43
Figure 7 - Dissociation of N-acetyl-L-phenylalanyl glycinal papain complex at pH 5.0 in the presence of varying amounts of the aldehyde	44
Figure 8 - The pH dependency for the dissociation of N-acetyl-L-phenylalanyl glycinal	46
Figure 9 - The pH dependency for the association of N-acetyl-L-phenylalanyl glycinal with papain	48

CHAPTER I

INTRODUCTION

Papain is a proteolytic enzyme isolated from the latex of papaya (carica papaya), the richest source being the juice of the fully grown green fruit. The term papain was first introduced by Wurtz and Bouchet in 1879 to describe the proteolytic principle in papaya latex. It is now recognized that the proteolytic activity of papaya latex is attributable to at least two distinct enzymes, papain and chymopapain (1). It is generally believed that papain is a representative member of a large class of proteolytic enzymes which contain a cysteine residue at their active site. Consequently, papain has been the subject of extensive investigation over the past forty years. The objective of much of this work has been to determine the catalytic mechanism of papain and thereby the mechanism of the entire class of cysteine proteinases. Papain is most frequently purified from dried papaya latex by extraction with 0.04 M cysteine at pH 5.7, sequential precipitation with ammonium sulfate and sodium chloride, and crystallization at 4°C from aqueous solutions containing 0.02 M cysteine at pH 6.5 (2). Crystals of papain prepared from either fresh or dried latex are minute needles. The needle like crystals change to larger hexagonal plates on standing for several months at low temperatures. The average yield of twice crystallized papain from good, dried latex is about 1 gram per 180 grams. The crystalline mercury derivative of papain, mercuripapain, is more stable and is prepared from papain by

recrystallization from 70% ethanol containing 1 mM HgCl_2 (3).

Mercuripapain prepared by the method of Kimmel and Smith, contains only about 0.5 mole of Hg per mole of enzyme (4). Mercuripapain may be further purified by affinity chromatography on a column containing either the peptide inhibitor Gly-Gly-Tyr(Bzl)-Arg or p-aminophenyl mercuric chloride covalently linked to agarose resin. The affinity purified, activated enzyme contains about 1 mole of SH group per mole of protein and usually exhibits about two to three times the specific activity of the original preparation (5).

Papain contains no chromophoric groups other than its constituent amino acids. It does not have any carbohydrate associated with it (6). Papain has the properties of a globulin. Isoelectric point (pI) of papain is 8.75. Molecular weight determined by sedimentation equilibrium and by amino acid sequencing are 23700 and 23406 respectively. Papain is very stable at high temperatures at near neutral pH. At acidic pH's (pH < 2) the protein is rapidly and irreversibly inactivated even at 25°C due to the unfolding of the molecule (2).

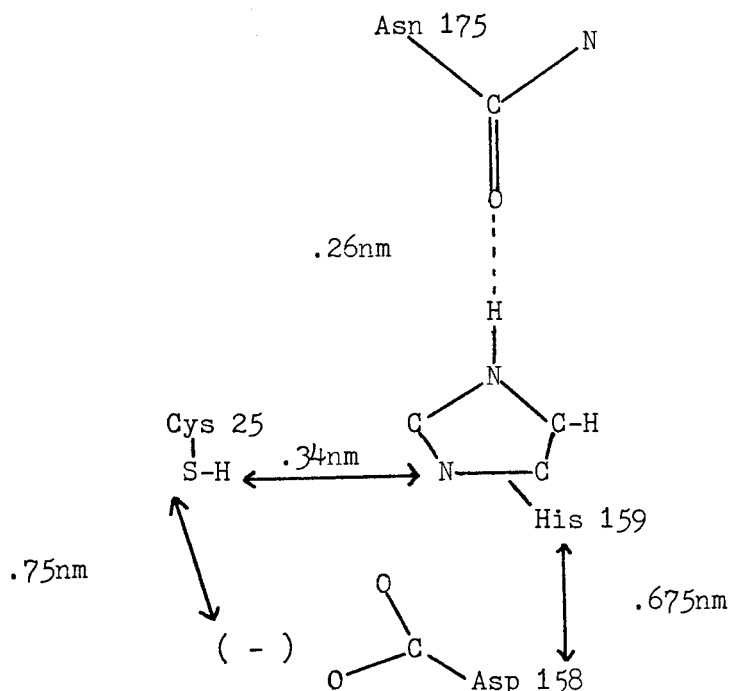
X-ray Studies

The amino acid sequence of this enzyme has been determined by a combination of chemical sequencing methods and X-ray diffraction studies (7). Drenth obtained an electron density map of papain at a resolution of 2.8 Å by a method of isomorphous replacement. The papain is roughly ellipsoidal with dimensions 36x48x36 Å. The X-ray studies shows the enzyme to be a single polypeptide chain of 212 amino acid residues folded into two independent domains, which are divided by a cleft. Each domain

is composed of about 100 amino acid residues. The active site region appears to be situated within the groove formed by the two halves of the molecule (8). The interaction between the enzyme and the substrate molecule occurs at the surface of the papain molecule in this groove. The side chain of the active site cysteine 25 is found in the groove. Close to the sulfhydryl group is the imidazole ring of histidine 159. There is only 3.4 Å distance between N-1 nitrogen of the imidazole side chain of histidine 159 and the sulfur atom of cysteine 25.

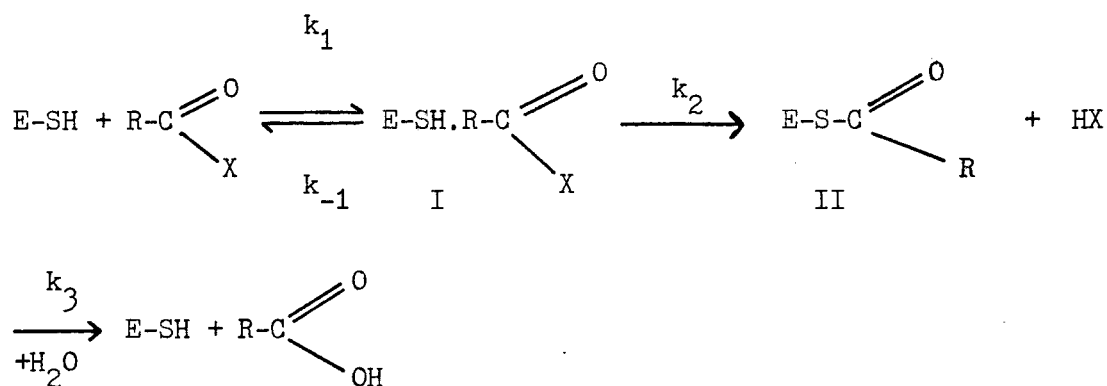
The imidazole ring of histidine 159 is hydrogen bonded through N-3 nitrogen to the side chain of Asn 175. Asn 175 is buried behind Trp 177. This tryptophan residue is also partially exposed to the solvent. Asp 158 is 6.7 Å away from His 159. Asp 64 is also situated in the groove. It is 10 Å away from the sulfur atom of Cys 25 and at 14 Å away from the imidazole ring of His 159. The active site is composed of approximately 40 residues from six different parts of the polypeptide chain.

The active site of papain is made up of seven subsites, each capable of accommodating a single amino acid residue of a peptide substrate. The size of the active site is about 25 Å so that each subsite is approximately 3.5 Å in length (9). The subsites are located on both sides of the catalytic site, four on the N-terminal side and three on the C-terminal side of the scissile peptide bond. The specificity of papain is determined in part by the specificity of these subsites.



Reaction of Papain with Substrates

The reaction of papain with substrates was shown to occur through the formation of an acyl enzyme intermediate(II) as shown below:



It has also been hypothesized that both the formation and decomposition of II occurs in two steps with the formation of additional intermediates. In these intermediates, the carbonyl carbon assumes a

tetrahedral configuration. One tetrahedral intermediate occurs during the acylation step k_2 and the other during the deacylation step k_3 .

Evidence for an Acyl Enzyme Intermediate

One of the evidences for an acyl enzyme intermediate (II) comes from stopped flow spectrophotometric studies of the pre-steady state reaction of papain with the o,m and p-nitrophenyl esters of carbobenzoxy glycine and the p-nitrophenyl ester of CBZ tyrosine (10). Thus, a burst of p-nitrophenol has been observed when CBZ-tyrosine p-nitrophenyl ester and papain are mixed together. The amount of p-nitrophenol released in this pre-steady state reaction is equal to the amount of the active enzyme present.

The steady state kinetics of papain catalyzed hydrolysis of p-nitrophenyl, benzyl and methyl esters of alpha-N-CBZ-L-lysine have also been studied as a function of pH and are consistent with the three step mechanism involving an acyl enzyme intermediate. In the case of these three substrates the acyl enzyme intermediates formed are identical. Since k_3 is the rate limiting step in the hydrolysis of these esters, k_{cat} should be similar for all three ester substrates. This was indeed found to be the case as shown in Table I.

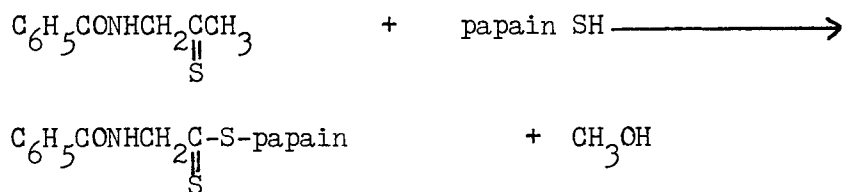
TABLE I

Kinetic Constant	Limiting Value			pK ₁ , pK ₂		
	PNPE	BE	ME	PNPE	BE	ME
k_{cat}, sec^{-1}	45.0 \pm 1.1	43.5 \pm 1.2	36.3 \pm 1.2	3.52	3.50	
$(k_{cat}/K_m) \times$ $10^5 \text{M}^{-1} \text{sec}^{-1}$	260 \pm 31	16.4	.155 \pm 0.11	4.30, 8.0	4.35, 8.35	8.53
k_3, sec^{-1}	45.9 \pm 1.6	45.0 \pm .6	46 \pm 2.5	3.33	3.30	
k_2, sec^{-1}	860	8.30 \pm 3.40	175 \pm 54	4.30 8.0	4.35 8.35	8.53
$K_m \times 10^3 \text{M}$	0.033	.52 \pm .08	10.7 \pm 2.6			

Limiting values of kinetic constants and corresponding pK values for the papain catalyzed hydrolysis of p-nitrophenyl, benzyl and methyl esters of Z-lysine.

An acyl enzyme intermediate has also been identified in the papain catalyzed hydrolysis of the non-specific substrate N-trans-cinnamoyl imidazole (11). On mixing equi-molar amounts of papain and N-trans-cinnamoyl imidazole, a transient intermediate appeared. The intermediate was sufficiently stable at pH 3.4 to be isolated by Sephadex chromatography. The intermediate had a UV absorption band at 325 nm. When cinnamoyl papain was denatured, the wave length of maximum absorption shifted to 309 nm. Since the possible model compound of trans-cinnamoyl papain, S-trans-cinnamoyl cysteine has a maximum absorbance of 306 nm, it was concluded that trans-cinnamoyl papain must be a thiol ester.

Lowe and Williams obtained strong evidence for an acylated thiol as an intermediate in papain catalyzed hydrolysis of methylthionhippurate (12).



III

This intermediate (III) is a dithioester and can be readily detected spectrophotometrically, since dithioesters show an intense absorption near 305 nm. It was observed that on addition of papain to a solution of methylthionhippurate, a strong absorption band at 313 nm appeared. The increase in absorption at 313 nm was thus in accord with the assumption that the dithio ester intermediate was formed, deacylation being the rate limiting step. These studies not only demonstrated the existence of an acyl enzyme intermediate, they also indicated that cysteine is the amino

acid residue which is the acyl acceptor. Therefore, the acyl enzyme intermediate is a thiol ester.

Role of the Individual Amino Acids at the Active Site

Cysteine 25

Papain requires an SH group like dithiothreitol for activation, a property which is characteristic of sulfhydryl enzymes. Papain is readily inactivated by oxygen, iodine, hydrogen peroxide and mercuric salts and can be reactivated with cyanide ions, hydrogen sulfide and thiols especially in the presence of EDTA (9). Reaction of papain with alkylating agents such as iodoacetic acid, iodoacetamide, and dibromoacetone results in complete loss of activity of the enzyme. The amino acid analysis of these alkylated papains demonstrate that cysteine 25 is modified in each case. From spectral evidence of acyl enzyme intermediate with thionohip-purate and N-trans-cinnamoyl imidazole, it was concluded that the acyl group is attached to the SH group of cysteine 25 and the function of cysteine 25 in catalysis is to act as the acyl acceptor.

Aspartic acid 158

From the pH dependency of the steady state second order rate constant, k_{cat} / K_m , for substrate hydrolysis by papain, it appears that two ionizable groups with pK's of 4.3 and 8.0 govern in this reaction (Table I). On the basis of this data, early workers concluded that the enzyme active site contained a carboxylate group in addition to the thiol group. By analogy with alpha chymotrypsin, they suggested that the role of the carboxylate was to act as a general base, extracting a proton from

the thiol group of cysteine during acylation. In deacylation the carboxylate group was thought to function as a general base extracting a proton from water or other nucleophiles. Another evidence for the participation of carboxyl group in substrate hydrolysis by papain comes from the work of Loffler (13). After chemical modification of a single carboxyl group with a water soluble carbodiimide and an amine, the enzyme was completely inactive.

While the X-ray studies also do show an aspartate at the active site, this residue is 7.5 \AA from sulfhydryl and it has been suggested that it is too far to be directly involved as a general base. The possibility still exists that while the aspartate may not participate directly in catalysis, its state of ionization may affect the activity of the enzyme by influencing the conformation of the active site or by some other mechanism. In addition, it has very recently been suggested that the active site of papain shows great conformational flexibility (38). As a consequence, the enzyme may be able to assume other conformations in solution in which aspartate 158 lies close to cysteine 25.

Histidine 159

Localization of a histidine residue within the active site region has been accomplished with the bifunctional reagent dibromoacetone. Amino acid analysis of the inhibited and performic acid oxidized enzyme clearly showed that a cysteine and a histidine residue within the active site region had been covalently linked with this bifunctional reagent. Furthermore, it was shown that the sites of alkylation were the sulfur of cysteine and the N-1 nitrogen of the imidazole ring of histidine. Thus,

these atoms must be within 5 \AA of each other in the tertiary structure of papain (14). It was further concluded that the histidine alkylated by dibromoacetone was histidine 159 (15).

X-ray crystallography has confirmed these results by showing the presence of a histidine residue at 3.4 \AA distance from cysteine 25. It was therefore suggested that the histidine and not aspartic acid acted as the general base in catalysis. But from pH dependency studies of k_2 and k_3 , a pK_a of 4.3 was assigned to the general base. Therefore, to accommodate the pH dependency data, chemical modification studies and x-ray crystallography data it was suggested that the active site histidine had an abnormally low pK.

Asparagine 175

X-ray data also showed the presence of an asparagine very close to histidine. It has been suggested that its function is to maintain the orientation and tautomeric state of the imidazole ring of histidine 159 (26).

Tryptophan 177

Residue I77 at the active site is tryptophan. X-ray data of Drenth suggested that side chains of histidine and tryptophan are close enough to permit an interaction between these groups and thus this residue may participate in the enzymatic reaction of papain. Tryptophan 177 appears to make a significant contribution to the fluorescence of papain. Protonation of histidine 159 quenches the fluorescence while binding of substrates and substrate analogs appears to enhance the fluorescence of tryptophan. Evidence for the presence of intramolecular histidine-

tryptophan complexes were obtained when flurometric titrations were conducted in aqueous solution in the range of pH 4 to 9. There were marked changes in fluorescence of the enzyme in this pH range both for mercuripapain as well as activated papain. However, the pK_a of the group quenching the fluorescence of tryptophan 177 was 8.6 in the active papain and 7 in the mercuripapain (16). In contrast, the pK_a of the fluorescence quenching group in S-thiomethyl papain is 4.3 (21).

Evidence for the pK's of the Active Site Residues

X-ray crystallography and chemical modification studies with dibromoacetone indicate that cysteine and histidine are in fact sufficiently close together to interact with each other. Therefore, it could be expected that both histidine and cysteine participate in catalysis. The studies of the pH dependency of the kinetic constant for papain catalysis has yielded the following results. When the pH rate profiles for the papain catalyzed hydrolysis of alpha N-benzoyl arginine ethyl ester and alpha N-benzoyl argininamide at 25°C was studied, it was found that both k_{cat} / K_m , which is equal to k_2 / K_s , and k_2 are dependent upon two ionizable groups with pK's of about 4.0 and 8.0, with the active form of the enzyme being the singly protonated species. When the pH dependency for the deacylation of alpha - N benzoyl-L-argininyl papain at 25°C was studied, it was found that k_3 is dependent on a single ionizable group with pK of about 4.0 with the active form of the acyl enzyme being unprotonated (17). On the basis of these results, early researchers proposed that the amino acid residue having a pK of about 8 and which participated in acylation only was the active site cysteine 25. The amino acid residue having a pK of about 4 and participating in both acylation and deacylation was

first identified as either aspartate or glutamate and latter as the active site histidine 159. It was also suggested that during acylation, attack by the thiol group of cysteine 25 on the carbonyl carbon of the substrate was facilitated by the imidazole side chain of histidine 159 acting as a general base. The usually low pK assigned to this histidine residue was attributed to an atypical environment.

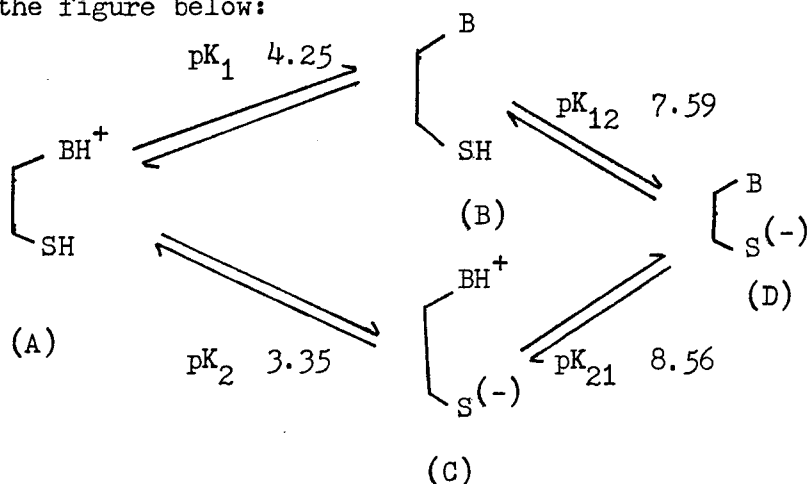
However, some evidence has been obtained to indicate that the pK of histidine side chain in papain is near normal. Studies of the pH dependency of the fluorescence of twice crystallized papain (18) have suggested that the pK of histidine side chain is greater than 7.0. Photo-oxidation studies have also indicated that the ionization of histidine may be near normal (19). In contrast the pK of histidine 159 side chain in S-thiomethyl papain determined by fluorometric titration is 4.3. These results suggest that the pK of histidine 159 may be linked to the chemical state of the sulfhydryl group of cysteine 25 (21).

Alternative Mechanisms for Papain Catalysis

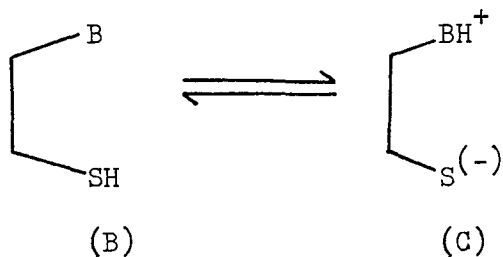
In 1972, Polgar studied the pH dependency and deuterium isotop effects on the rate of reaction of the active site SH group of papain with chloroacetamide (20). In this reaction, a double sigmoidal pH rate profile was obtained with pK's 4.0 and 8.5. No deuterium isotope effect was observable on the rate constants of the alkylation reaction. At higher pH's, the rate of alkylation of the SH group becomes faster by several orders of magnitude over that of a normal SH group. The significant observation made by Polgar was that the singly protonated form of papain possessed considerable reactivity toward the alkylating reagent.

From this data he concluded that at intermediate pH's the active form of papain contained both neutral thiol and histidine residues at its active site and that the action with the alkylating reagent occurred by a rapid transfer of the proton from the thiol to the histidine forming a thiolate imidazolium ion pair. The thiolate anion then reacted in a slow step with the chloroacetamide. At high pH the active form of the enzyme, containing a thiolate anion and a neutral imidazole, papain reacted directly with the alkylating agent.

Lewis and co-workers studied the pH dependency of the proton uptake and release during modification of the active site cysteine residue of papain. From these data they could calculate values for three of the four microscopic ionization constants for the active site cysteine and histidine residues provided they could independently measure one of the four constants (21). They assigned pK_1 a value of 4.25 on the basis of the results of a spectrophotometric titration of the histidine residue of papain-S-SCH₃. The calculated values of pK_2 , pK_{12} , pK_{21} are shown in the figure below:



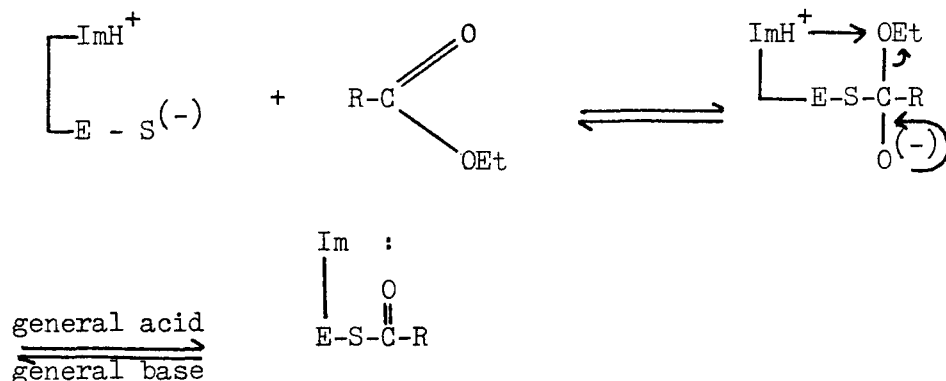
When the sulfur of cysteine 25 is neutral the histidine seems to have an abnormally low pK. When the SH group is ionized or otherwise bears a negative charge, the histidine has a somewhat higher than normal pK. Conversely, when the histidine residue is protonated the SH group has a near normal pK. The equilibrium constant calculated for



the interconversion of species B and C indicate that the thiolate-imidazolium ion pair already predominates at pH's where papain is active.

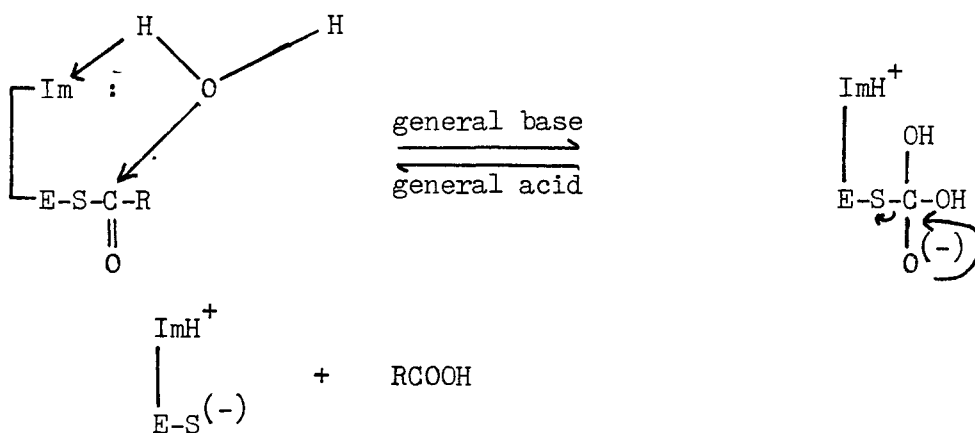
It may now be possible to propose an alternative mechanism for papain catalysis based on the works of Lewis and Polgar. Accordingly, acylation involves an unfacilitated attack of the thiolate anion on the carbonyl carbon of the ester to form a tetrahedral adduct. Decomposition of this intermediate with the removal of an alcohol or an amine may be facilitated by histidine acting as a general acid (20, 37).

Acylation:



Since deacylation can be considered to be similar to the microscopic reverse of acylation, this step could involve general base catalyzed attack of water on the carbonyl carbon of the acyl enzyme to form a second tetrahedral intermediate. Decomposition of this tetrahedral intermediate with the cleavage of carbon sulfur bond would be unfacilitated.

Deacylation:



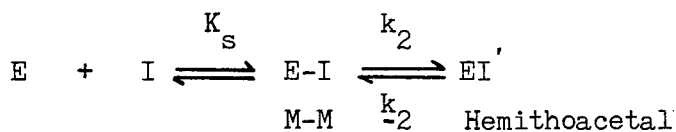
The catalytically active form of the enzyme is favored in each step since in acylation (sulfur negatively charged) the histidine has a high pK while in deacylation (sulfur neutral) the pK of histidine is abnormally low. These conclusions are consistent with the fluorometric titration data which suggests a high pK_a for histidine in free papain and a low pK_a in $S-SCH_3$ modified papain.

Reaction of Papain with Aldehydes

It has been discovered that aldehydes are very strong inhibitors of papain. Westerik and Wolfenden found that the K_I for N-acetyl-L-phenylalanyl glycinal was 4.6×10^{-8} M. This aldehyde is believed to combine with the active site cysteine residue of the enzyme to form a hemithioacetal that resembles the proposed tetrahedral intermediate (22).

Evidence for hemithioacetal formation has been obtained by Bendall et al using cross saturation technique in NMR spectroscopy (23). The loss of aldehydic proton signal in N-acetyl amino acetaldehyde when a solution containing papain and aldehyde was irradiated with a high power radio frequency source in the region of 4.0ppm (where the hemithioacetal absorbs) is consistent with the existence of an enzyme-hemithioacetal in equilibrium with free enzyme and free aldehyde. Although the chemical shift at which Hz causes cross saturation of the aldehydic proton signal to occur is below that expected for a simple hemithioacetal, its total magnetic environment within the enzyme active site could account for this. The coupling constant for the bound species is found to be 173 Hz which is in the expected region for a hemithioacetal.

Another evidence for the formation of a hemithioacetal adduct has come from the work of Mattis et al (24). The fluorescence emission intensity of tryptophan 177 increases when aldehyde is bound to papain. The increase in fluorescence is observed to occur in two steps. First there is a rapid increase in fluorescence which is attributed to the formation of a Michelis Menten complex. The rapid increase is followed by a slow first order increase which is presumed to represent the conversion of Michelis Menten complex to the hemithioacetal adduct.



when $k_2 \gg k_{-2}$

$$k_{\text{obs}} = \frac{k_2 (\text{Aldehyde})}{K_s + (\text{Aldehyde})}$$

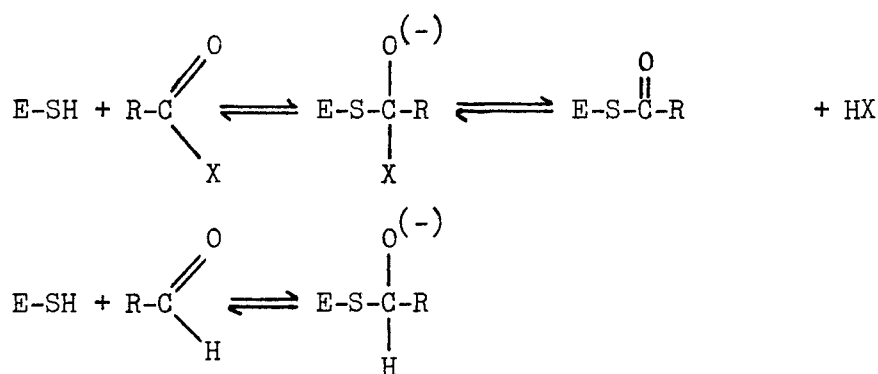
In addition, the inhibitor constant, K_I , obtained from steady state measurements can be shown to be related to these kinetic constants by the following equation:

$$K_I = \frac{k_{-2}}{k_2 + k_{-2}} \times K_s$$

Fruton determined the values for k_2 and K_s using a stopped flow spectrophotofluorometer. K_I was measured by studying aldehyde inhibition of steady state hydrolysis of N-acetyl phenylalanyl glycine-p-nitroanilide. From the equation $K_I = \frac{k_{-2}}{k_2 + k_{-2}} \cdot K_s$, he estimated k_{-2} . The estimated

value for K_s is $70 \times 10^{-6} M$, k_2 is 10 sec^{-1} , K_I is $1 \times 10^{-8} M$ and k_{-2} is 0.0014 sec^{-1} . According to these results, the dissociation of the enzyme from the aldehyde is a very slow reaction.

The high stability of this hemithioacetal adduct in papain is thought to be due to the fact that it resembles the intermediate on the pathway of enzyme catalysis. A comparison of the acylation reaction to hemithioacetal formation can be shown as follows:



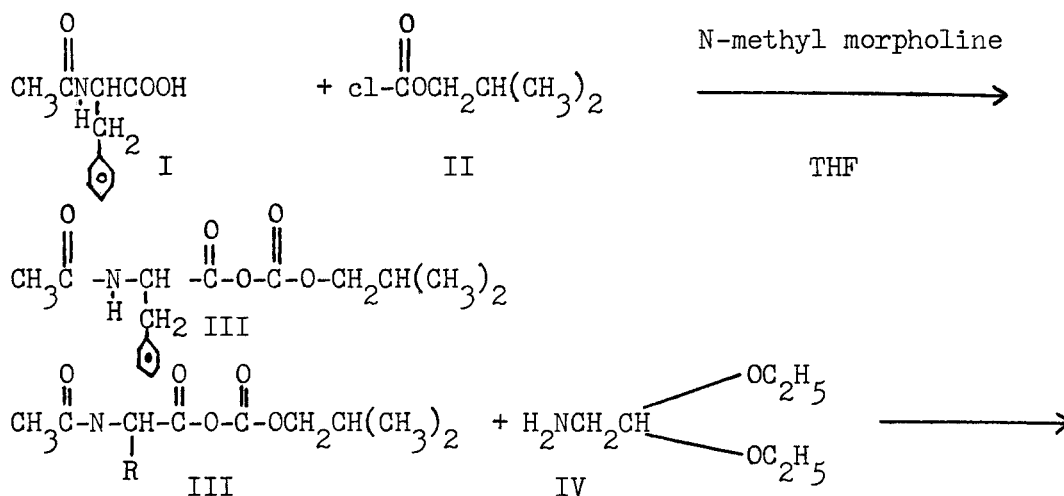
Hemithioacetal formation may thus be a good model for the formation of the proposed tetrahedral intermediate in the acylation of papain by substrates. Studies of hemithioacetal formation permit one to isolate and study an early step in the reaction of papain with substrates. Information obtained from the study of the mechanism of reaction of aldehydes with papain may therefore, allow one to determine which of the various mechanisms proposed for papain catalysis is correct.

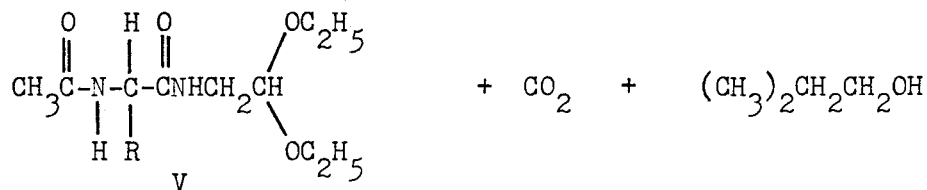
CHAPTER II

MATERIALS

Mercuripapain and Benzyloxycarbonyl glycine p-nitrophenyl ester were obtained from Sigma Chemical Company. For the fluorescence experiments, the enzyme was purified on a mercurial Sepharose affinity column by the method of Sluyterman et al (31). The inhibitor N-acetyl-L-phenylalanylglycinal diethyl acetal was synthesized in this laboratory from N-acetyl L-phenylalanine and aminoacetaldehyde diethyl acetal (27). Generation of the free aldehyde from the acetal was accomplished by incubation of the acetal with $10^{-2}M$ HCl at $25^{\circ}C$ for 24 hours (22). Sodium tetrathionate, the trapping agent for the enzyme in the studies of the dissociation of the enzyme aldehyde complex, was recrystallized from ethanol.

Synthesis of N-Acetyl-L-Phenylalanyl glycinal





A solution of 2.5m moles of N-acetyl phenylalanine (I) was dissolved in tetrahydrofuran (previously dried over magnesium sulfate). It was chilled to -15°C with stirring in a CCl_4 dry ice bath. Then 2.5 moles of N-methyl morpholine was added, followed by 2.63 mmoles of isobutyl chloroformate (II). Thirty seconds after the addition of isobutyl chloroformate 2.63 mmoles of aminoacetaldehyde diethyl acetal (IV) was added. After several minutes, the bath was removed and the solution was allowed to come to room temperature for half an hour. The reaction mixture was then concentrated under vacuum. The residue was taken up in a solution containing 15ml chloroform, 85ml ethyl acetate and 10ml water. After shaking, water layer was removed and the organic layer was washed with 10ml of saturated sodium bicarbonate solution and then 10ml of water. The organic phase was dried over anhydrous magnesium sulfate and evaporated under vacuum. The product (V) was then recrystallized from ethyl acetate. The product yielded a single spot on TLC. Its melting point was $130-131^{\circ}\text{C}$. Analysis of N-acetyl-L-phenylalanyl glycinal diethyl acetal, by Galbriath Laboratories, Knoxville, Ten. gave the following result:

	%C	%H	%N	Difference (%O)
Calculated	63.33	8.13	8.69	19.85
Found	63.23	8.16	8.32	20.29

Synthesis of Organomercuri Sepharose 4B

200g of Sepharose 4B gel was well washed with water on a sintered glass filter and suspended in 200ml water. A solution of 8g of cyanogen bromide in 320ml of water was also prepared. In the hood, the CNBr solution was added to Sepharose and the pH was raised to 11 by addition of 2M NaOH with constant stirring. The pH was maintained for six minutes by addition of more NaOH. The gel was then washed with a large volume of .1M NaHCO_3 at 4°C . The activated Sepharose kept at 4°C , was suspended in 400ml DMSO 10%(v/v) and 0.52g of aminophenyl mercuric acetate, dissolved in 4ml of DMSO, was introduced slowly and with stirring. The mixture was stirred at 4°C overnight. The gel was then transferred to a chromatographic column and washed with 20% DMSO. After washing with aqueous DMSO, the column was washed with sodium acetate buffer pH 5.5 to remove DMSO (29, 30, 31).

Affinity Purification of Papain

Preparation of Affinity Column

The mercurial Sepharose 4B prepared as described above was placed in a small column having a volume of about 15ml. This column was washed with several bed volumes of 0.05M cysteine followed by 5 bed volumes of buffer (0.05M NaAc, 0.1M NaCl pH 5.0) to remove excess thiol. Bound thiol was then removed by washing the column with 10 bed volumes of buffer (0.05M NaAc, 0.1M NaCl, 0.002M HgCl_2). The column was then regenerated by washing with buffer (0.05M NaAc, 0.1M NaCl) until all free mercury was removed as determined by the dithizone test. The test was performed by adding to 1ml of column effluent, 10 micro-liter of a 0.5% dithizone

solution in CCl_4 and 1ml of CCl_4 . The mixture was then vortexed vigorously. If the CCl_4 phase remained green in color, then the column was assumed to contain no more free mercury. The column was then equilibrated with 1.5 column volumes of 0.1M NaPi pH 8.0.

Determination of Column Capacity

To the column was added a solution containing 2.5mM dithionitro benzoic acid and 15mM Na_2SO_3 in 0.1M NaPi pH 8.0 until the entire column was stained yellow (at least one column volume). The column was then washed with 0.1M NaPi to remove unbound thionitrobenzoic acid (about 1-2 column volumes). The column was then washed with one column volume of 0.05M acetate, 0.1M NaCl and 2.0mM HgCl_2 . An aliquot of the effluent was added to a 0.1M solution of L-cysteine at pH 8.5 and the color was measured at 412nm. From the color yield and the volume of the effluents, the capacity of the column can be calculated. The capacity of the resin was 1.4×10^{-2} $\mu\text{moles-SH}$ group bound per milliliter Sepharose.

Papain Purification

The mercurial Sepharose was washed with 0.05M NaAc and 0.1M NaCl at pH 5.0 to remove soluble mercury. The enzyme was activated with cysteine, filtered through a sephadex G 25 column to separate the activated enzyme from excess activator. Activated papain free of activators was applied to the column. The nonbinding portion was eluted with buffer containing 0.05M NaAc, 0.1M NaCl pH 5.0. Bound papain was then eluted with approximately 25 column volumes of 0.05M NaAc, 0.1M NaCl, 0.5mM HgCl_2 pH 5.0. The effluent was assayed and the tubes containing enzyme were pooled and concentrated using an Amicon Concentrator.

CHAPTER III

METHODS

The K_I for N-acetyl-L-phenylalanylglycinal. was determined with CBZ-glycine p-nitrophenyl ester as a substrate. The reaction was performed under conditions where $K_m \gg S$. This necessitated the use of 10cm cuvettes. Substrate hydrolysis was monitored at 346.5nm with a Cary 15 recording spectrophotometer thermostated at $25^\circ\text{C} \pm 0.2^\circ\text{C}$.

Measurements of K_I for N-Acetyl-L-Phenylalanyl Glycinal

In a preliminary experiment, buffer, preactivated enzyme and aldehyde were incubated for various time periods and the reaction was initiated by the addition of substrate. Minimum incubation period required for complete inhibition was 16 minutes. Requirement of this incubation period seemed more important at lower pH's. On this basis, the following procedure was adopted for measuring K_I .

To a cuvette (10cm light path) was added 25ml buffer which had been previously equilibrated at 25°C in a water bath. In the pH range from 6.0 to 8.0 the buffer was 0.05M phosphate, 0.2M NaCl, $5 \times 10^{-4}\text{M}$ EDTA; in the pH range 4.0 to 5.5, the buffer was 0.05M acetate, 0.2M NaCl, $5 \times 10^{-4}\text{M}$ EDTA; and at pH 3.5 the buffer was 0.05M formate 0.2M NaCl, $5 \times 10^{-4}\text{M}$ EDTA. To the appropriate buffer was then added, an aliquot of preactivated enzyme such that the final concentration of the enzyme varied from about $7 \times 10^{-9}\text{M}$ to $6 \times 10^{-8}\text{M}$. For the inhibition reactions, varying amounts of inhibitor were added to give inhibitions which ranged from 25 to 75%. This

required final concentrations of N-acetyl-L-phenylalanylglycinal which varied from $6 \times 10^{-9} \text{M}$ to $1 \times 10^{-7} \text{M}$. After incubating the buffer, enzyme and aldehyde for 16 minutes 25 microliter of CGN (in acetonitrile) was added with thorough mixing to initiate the reaction. Final concentration of CGN was $1.1 \times 10^{-7} \text{M}$ in all experiments. The rate for the uninhibited reaction was determined in absence of inhibitor. The entire reaction was followed to completion. The first order rate constants k_1 and $k_1(I=0)$ were obtained from plots of $\ln (A - A_t)$ against time.

Enzyme Assay

Papain was preactivated in a small test tube by adding a small volume of a solution containing an excess of L-cysteine to an aliquot of a stock solution of HgPapain. To a cuvette was then added 3ml of acetate buffer pH 5.0 (0.05M NaAc, 0.2M NaCl, $5 \times 10^{-4} \text{M}$ EDTA) and 50 microliter of preactivated papain solution approximately $1.7 \times 10^{-5} \text{M}$. The reaction was then initiated by the addition of 25 microliter of CGN ($5.7 \times 10^{-5} \text{M}$) and the solution thoroughly mixed. The increase at 346.5nm was measured in a GCA/McPherson Double beam spectrophotometer thermostated at $25 \pm 0.2^\circ \text{C}$. Reaction rates were expressed as the change in absorbance at 346.5nm per minute.

Fluorescence Studies

The fluorescence emission and excitation spectra of papain were obtained with an Aminco Bowman spectrophotofluorometer. The excitation and emission maxima were found to be 288nm and 344nm. The fluorescence of aldehyde bound papain was several fold greater than that of free enzyme. The rate of decomposition of the enzyme-aldehyde complex was therefore

followed spectrophotofluorometrically at excitation and emission wavelengths of 288 and 344nm respectively. The cell compartment was thermostated at $25 \pm 0.5^\circ\text{C}$.

Measurement of Dissociation of Papain Aldehyde Complex

To a 1cm fluorometer cuvette was added 35microliter of N-acetyl-L-phenylalanyl glycinal (final concentration $2.3 \times 10^{-7}\text{M}$), affinity purified mercuripapain (concentration about $2 \times 10^{-7}\text{M}$) and dithiothreitol (final concentration approximately $1.2 \times 10^{-4}\text{M}$). To this was added 3ml of an appropriate buffer with mixing and the fluorescence was measured. Between pH 6.0 and 7.5 the buffer was 0.05M phosphate, 0.2M NaCl, $5 \times 10^{-4}\text{M}$ EDTA; between pH 3.0 and 5.5 the buffer was 0.05M acetate, 0.2M NaCl, $5 \times 10^{-4}\text{M}$ EDTA. Once a stable baseline was obtained, various amounts of tetrathionate were added such that its concentration ranged from 6.2×10^{-4} to 9.3×10^{-3} . The decrease in fluorescence was recorded with time. The entire reaction was recorded until an endpoint was reached.

Measurement of the pH Dependency of the Fluorescence of Papain and Aldehyde Bound Papain

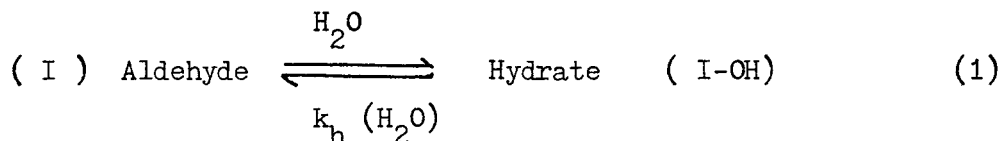
To a fluorometer cuvette was added 25microliter mercuripapain ($2.7 \times 10^{-5}\text{M}$), 200microliter of dithiothreitol (0.06M) and 3ml of appropriate buffer. The solution was mixed thoroughly and the fluorescence of this solution was measured. Between pH 6.0 and 9.5 the buffer was 0.05M phosphate, 0.2M NaCl, $5 \times 10^{-4}\text{M}$ EDTA; between pH 3.0 and 5.5 the buffer was 0.05M acetate, 0.2M NaCl, $5 \times 10^{-4}\text{M}$ EDTA and below pH 3.0 the buffer was 0.05M formate, 0.2M NaCl, $5 \times 10^{-4}\text{M}$ EDTA. To obtain the fluorescence of the aldehyde complex 100microliter of aldehyde ($4.3 \times 10^{-3}\text{M}$) was then added and

the fluorescence remeasured. The final concentration of aldehyde was always much greater than its K_I .

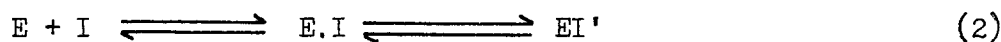
CHAPTER IV

KINETIC DERIVATIONS

We wished to investigate the binding of N-acetyl-L-phenylalanyl glycinal to the proteolytic enzyme papain as a function of pH. Although N-acetyl-L-phenylalanyl glycinal exist predominantly in its hydrated form in aqueous solution, it is believed that only the free aldehyde is an effective inhibitor (23). When in solution, the aldehyde and its hydrate are in equilibrium with each other according to the following relationship.



If we further assume that only the unhydrated aldehyde can react with papain, then we can write for this reaction



According to this equation, the aldehyde first binds to the enzyme to form a Michelis Menten complex (E.I). Then this complex will be converted to the hemithioacetal adduct (EI'). K_I' , the apparent equilibrium dissociation constant is given by the following equation. In this equation, (I) is the concentration of unhydrated aldehyde.

$$K_I' = \frac{(\text{E}) (\text{I})}{(\text{EI}) + (\text{EI}')} \quad (3)$$

Since free aldehyde is related to total aldehyde by the relationship $(I) = (I_o) / (1+K_h)$, we can define K_I in terms of total aldehyde concentration.

$$K_I = K'_I (1+K_h) = \frac{(E) (I_o)}{(EI) + (EI')} \quad (4)$$

Under conditions where $S_o \ll K_m$ and in the presence of a strongly binding inhibitor, the following equation of Perlstein and Kezdy describes the relationship between inhibitor concentration and pseudo first order rate constant for substrate hydrolysis (46):

$$k_i = \frac{k_o}{1 + \frac{K_I + (K_m/k_{cat}) k_i}{(I_o)}} \quad (5)$$

In this equation, k_o is the pseudo first order rate constant in the absence of inhibitor.

$$k_o = (k_{cat}/K_m) (E_o) \quad (6)$$

k_i is the pseudo first order rate constant in the presence of inhibitor

$$k_i = (k_{cat}/K_m) (E_{free}) \quad (7)$$

K_I is the equilibrium dissociation constant described by equation 4.

Equation 5 may be rearranged as follows

$$k_o - k_i = \frac{k_i (I_o)}{K_I + (K_m/k_{cat}) k_i} \quad (8)$$

From equation 6 it can be shown that

$$(K_m/k_{cat}) k_i = (k_i/k_o) (E_o) \quad (9)$$

Substituting equation 9 into equation 8, rearranging and multiplying both sides of equation by k_o/k_i we have

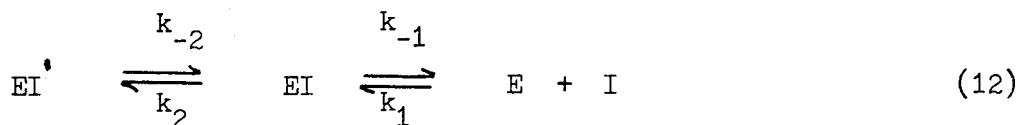
$$k_o (I_o) / k_o - k_i = K_I (k_o/k_i) + E_o \quad (10)$$

Equation 10 may be further rearranged

$$\frac{I_o}{1 - (k_i/k_o)} = \frac{K_I}{k_i/k_o} + E_o \quad (11)$$

Plotting $\frac{I_o}{1 - (k_i/k_o)}$ vs $\frac{1}{k_i/k_o}$ should yield a straight line with a slope of K_I and an intercept of (E_o) . Thus K_I can be determined from the slope of the plot. Furthermore, it can be seen that strongly binding inhibitors such as the aldehydes may be used to titrate papain solutions since (E_o) can be obtained.

Because the enzyme aldehyde complex is in equilibrium with free enzyme and free aldehyde, it is possible to dissociate this tight complex in the presence of reagents which can trap either the free enzyme or free aldehyde. The reaction of the EI complex with a reagent that can react with either free enzyme or free aldehyde is shown below for the condition where $K_s \gg I$.



If either step 13 or 14 is very fast and if k_{-1} is faster than k_2 , the rate of disappearance of EI' will be equal to the rate constant for the dissociation of aldehyde from enzyme, k_{-2} .

When $K_S \gg I$ or if it is able to react with T , EI in equation 2 and 3 will not be present in significant amounts. Then the following equality will hold true.

$$-\frac{d(EI')}{dt} = k_{-2}(EI') - (k_2/K_S)(E)(I) \quad (15)$$

Also, when the trapping reagent reacts with enzyme,

$$\frac{d(E)}{dt} = k_{-2}(EI') - k_2/K_S(E)(I) - k_T(E)(T) \quad (16)$$

Making the steady state assumption for E , $\left|\frac{d(E)}{dt}\right| \lll \left|\frac{d(EI')}{dt}\right|$ have

$$(E) = \frac{k_{-2}}{k_2/K_S(I) + k_T(T)} \quad (17)$$

Substituting (17) into (15) and rearranging we have

$$\frac{-d(EI')}{dt} = \frac{k_{-2}(EI')}{(k_2/K_S)(I) + k_T(T)} \quad (18)$$

Since $I_o = I + EI'$, $I = I_o - EI'$ and

$$-\frac{d(EI')}{dt} = \frac{k_{-2}(EI') k_T(T)}{(k_2/K_S)(I_o - EI') + k_T(T)} \quad (19)$$

According to this equation, the decomposition of (EI') in the presence of a trapping agent may not be strictly first order. This process will be first order under the following conditions.

When $I_o \gg EI'$, and $T \gg E$

$$-\frac{d(EI')}{dt} = \frac{k_{-2}(EI') k_T(T)}{(k_2/K_S)(I_o) + k_T(T)} \quad (20)$$

$$\ln(EI') = - \left[\frac{k_{-2} k_T(T)}{(k_2/K_S)(I_o) + k_T(T)} \right] t + C \quad (21)$$

In equation 21,

$$k_{obs} = \frac{k_{-2} k_T(T)}{(k_2/K_S)(I_o) + k_T(T)} \quad (22)$$

$$\text{Thus, } 1/k_{obs} = \frac{(k_2/K_S)(I_o)}{k_2 k_T(T)} \quad (23)$$

A plot of $1/k_{obs}$ vs $1/T$ yields a straight line with a slope equal to

$$\frac{k_2}{k_{-2} K_S} \frac{(I_o)}{K_T} \text{ and an intercept equal to } 1/k_{-2}.$$

Similarly a plot of $1/k_{obs}$ vs (I_o) yields a straight line with a slope equal to $\frac{k_2}{k_{-2} K_S} \frac{1}{k_t(T)}$ and an intercept equal to $1/k_{-2}$.

When $k_T(T) \gg (k_2/K_S)(I)$, $k_{obs} = k_{-2}$.

CHAPTER V

RESULTS

Preparation of N-Acetyl-L-Phenylalanyl glycinal

The diethylacetal of N-acetyl-L-phenylalanyl glycinal was prepared from N-acetyl-L-phenylalanyl glycinal and aminoacetaldehyde diethylacetal by the mixed anhydride procedure of Anderson et al (27). This method is reported to cause no significant racemization about the alpha-carbon of N-substituted amino acids. The free aldehyde was then generated by hydrolysis of the diethylacetal in dilute aqueous HCl as described by Westerik and Wolfenden (22).

Dissociation Constants for N-Acetyl-L-Phenylalanyl glycinal with Papain

The Michelis-Menten rate equation can not be used to calculate a dissociation constants for inhibitors which bind strongly to enzymes. For a strongly binding competitive inhibitor equation 11 describes the relationship between the pseudofirst order rate constants for substrate turnover in the presence (k_i) and absence ($k_{i(I=0)}$) of inhibitor and the inhibitor of dissociation constant (K_I) (46).

$$\frac{I_o}{1-(k_i/k_o)} = \frac{K_I}{k_i/k_o} + (E_o) \quad (11)$$

In order to determine K_I the hydrolysis of CGN by papain was monitored spectrophotometrically under conditions of $K_m \gg (S)$ in the absence and

presence of various concentrations of inhibitor. The first order rate constants, k_1 , were obtained by a graphical analysis of the absorbance data. Typical first order plots of such absorbance data are shown in figure 1. The first order rate constants obtained from the slopes of these lines are plotted according to equation 11 in figure 2. The best straight line, drawn through the data points the method of least squares, yielded a value for K_I at pH 6.5 of $8 \times 10^{-9} M$ in reasonable agreement with values of $1 \times 10^{-8} M$ and $4.6 \times 10^{-8} M$ obtained previously by Mattis et al (24) and by Westerik and Wolfenden (22). The values of (E_0) obtained from the intercept at pH 5 indicated that preparations of HgPapain used in these studies contained 30 to 40% active enzyme, in agreement with estimates of purity obtained by titration of L-Cysteine activated HgPapain with DTNB. Table II contains the values for K_I calculated by this method from data obtained between pH 3.5 and 8.0. These values for K_I are uncorrected for aldehyde hydration. In Figure 3, the corresponding association constants, $1/K_I$, are plotted as a function of pH. As can be seen, the association constant decreases progressively with decreasing pH below pH 5.0. In this figure, the points represent the experimentally determined values of $1/K_I$ along with their standard error and the solid line was calculated for a group on the enzyme with a pK_a of 4.25 according to the relationship

$$(1/K_I)_{\text{obs}} = \frac{(1/K_I)_{\text{lim}}}{1 + (H)/K_a} \quad (25)$$

Figure 1. Inhibition of Papain catalyzed hydrolysis of CGN by N-acetyl-L-phenylalanyl glycinal at $25 \pm 0.2^{\circ}\text{C}$. The buffer was 0.05M acetate, $5 \times 10^{-4}\text{M}$ EDTA and 0.2M NaCl, pH 5. The substrate concentration was $1.1 \times 10^{-7}\text{M}$ and enzyme concentration $1.92 \times 10^{-8}\text{M}$. The aldehyde concentrations were curve (1) 0; curve (2) $6.31 \times 10^{-9}\text{M}$; curve (3) $1.387 \times 10^{-8}\text{M}$; curve (4) $2.65 \times 10^{-8}\text{M}$.

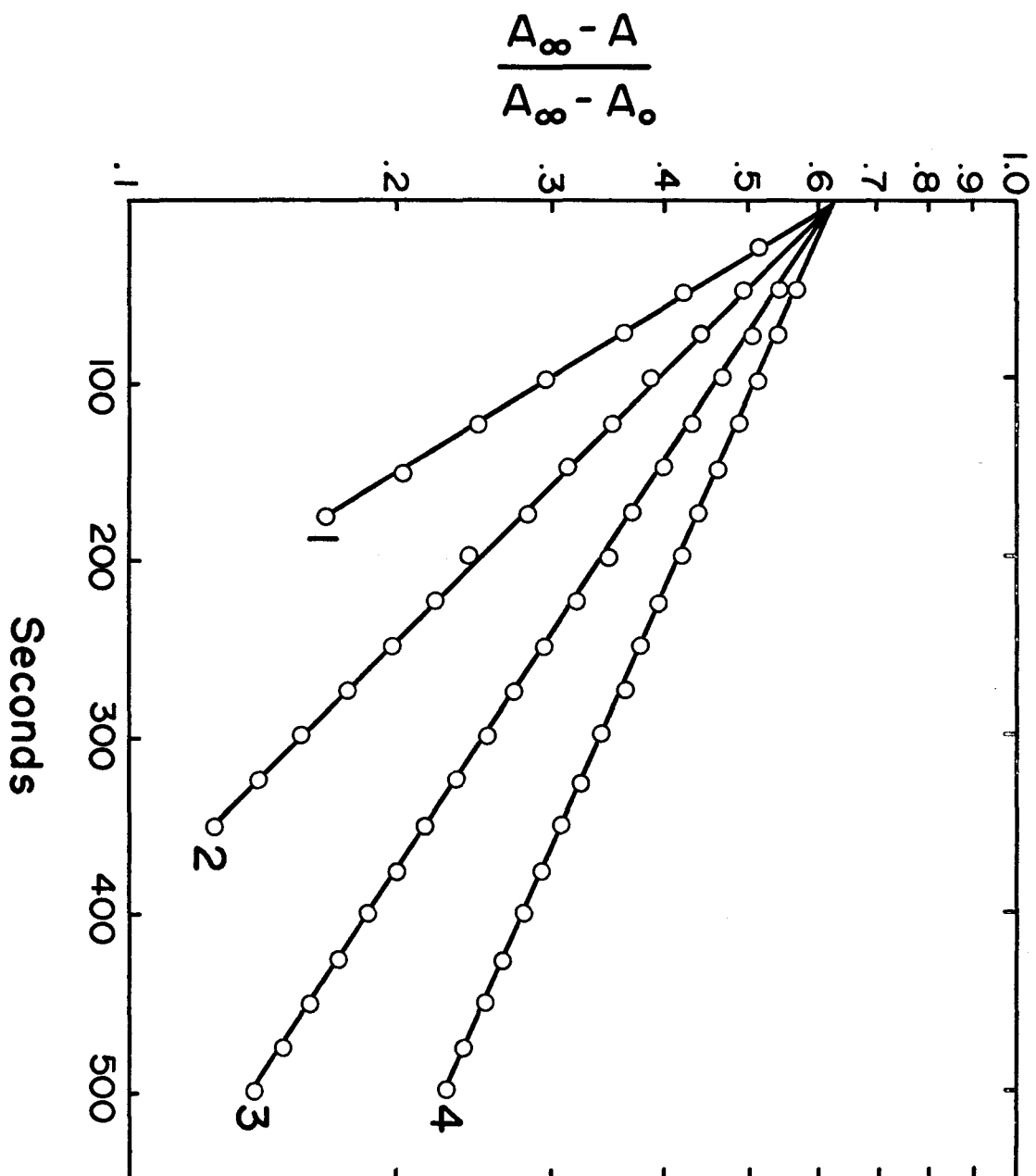


Figure 2. Determination of the equilibrium dissociation constant for N-acetyl-L-phenylalanyl glycinal with papain at pH 5.0 and $25 \pm .2^{\circ}\text{C}$. First order rate constants, such as obtained from Figure 1, were plotted according to the equation of Kezdy and Pearlstein as described in methods. The slope of the line gave a value for K_I of $9.26 \times 10^{-9} \text{M}^{-1}$ and the intercept gave a value for the enzyme concentration of $1.83 \pm .37 \times 10^{-8} \text{M}$.

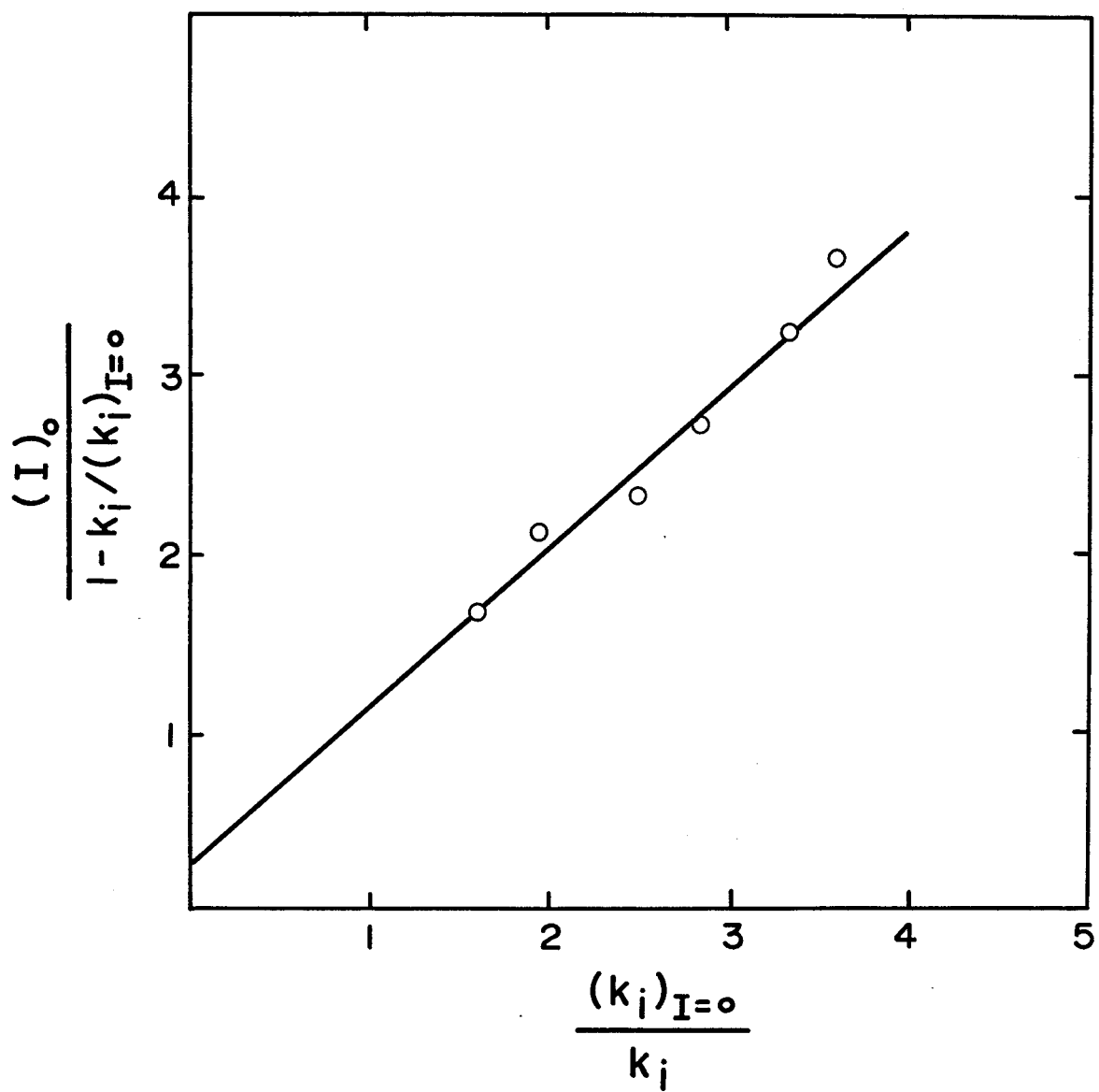
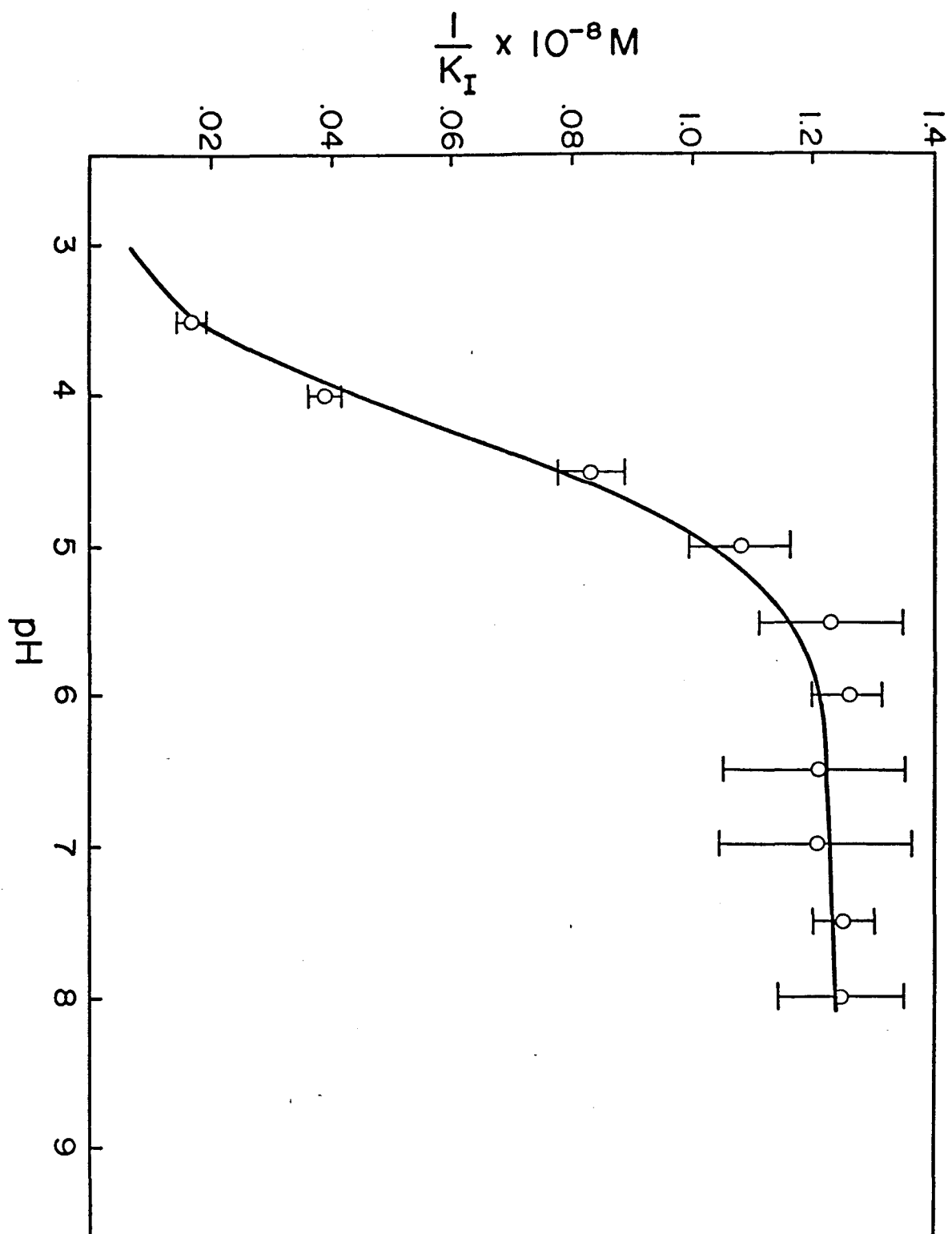


TABLE II

The pH dependency of the equilibrium dissociation constant for N-acetyl-L-phenylalanyl glycinal with papain

pH	$K_I \text{ (M)} \times 10^9$
3.5	62.5 ± 4.1
4.0	25.6 ± 1.8
4.5	12.0 ± 0.5
5.0	9.3 ± 0.7
5.5	8.3 ± 0.7
6.0	8.0 ± 0.2
6.5	8.3 ± 1.1
7.0	8.3 ± 1.2
7.5	8.0 ± 0.3
8.0	8.0 ± 0.7

Figure 3. The pH dependency of the equilibrium association constant, $1/K_I$, for N-acetyl-L-phenylalanyl glycinal with papain at $25 \pm .2^\circ\text{C}$. Buffers used at each pH are described in experimental section. K_I were obtained as described in Figure 2.



Fluorescence of Affinity Purified Papain and its N-Acetyl-L-Phenylalanyl Glycinal complex

The binding of dipeptide aldehyde analogue to papain at pH 6.5 has been reported to result in a significant increase in the native fluorescence of this enzyme (24). When these experiments were repeated with commercial preparations of Hg papain, activated with either L-cysteine or mercaptoethanol, only a small fluorescence increase was observed. In contrast, the binding of N-acetyl-L-phenylalanyl glycinal to papain purified by affinity chromatography on Phenylmercuri-Sepharose resulted in a large enhancement in the enzyme's fluorescence. This is shown in Figure 4. Thus at an excitation wavelength of 288nm, the binding of the dipeptide aldehyde to papain results in a 3 fold increase in the fluorescence emission of the enzyme at 344nm at pH 6.5.

Figure 5 shows the pH dependency of the intensity of the fluorescence emission at 344nm of activated mercuri-Sepharose purified papain in the absence and presence of aldehyde. To insure saturation of the enzyme by aldehyde, an aldehyde concentration was chosen such that the relationship $(I) \gg (E) \gg K_I$ was obeyed at all pH's studied. Since K_I was not measured below pH 3.5 and above pH 8.5, it was calculated in those pH ranges using equation (25). The fluorescence of the free aldehyde at 344nm was also determined under these conditions and was found to make no measurable contribution to the fluorescence of the complex.

The pH dependency of the fluorescence of active, mercuri-Sepharose purified papain, lower curve, appears to be determined in large part by the ionization of two amino acid residues. The solid line (in the lower

Figure 4. Fluorescence Emission spectra of papain in the presence and absence of N-acetyl-L-phenylalanyl glycinal at pH 6.5. The buffer was 0.2M phosphate containing 1×10^{-4} M EDTA. The concentration of papain and mercaptoethanol were 2.4×10^{-7} M and 2.5×10^{-3} respectively. Curve 2 was obtained by adding to N-acetyl-L-phenylalanyl glycinal to the fluorometer cell at a final concentration of 3.1×10^{-6} M. The excitation wavelength was 288nm.

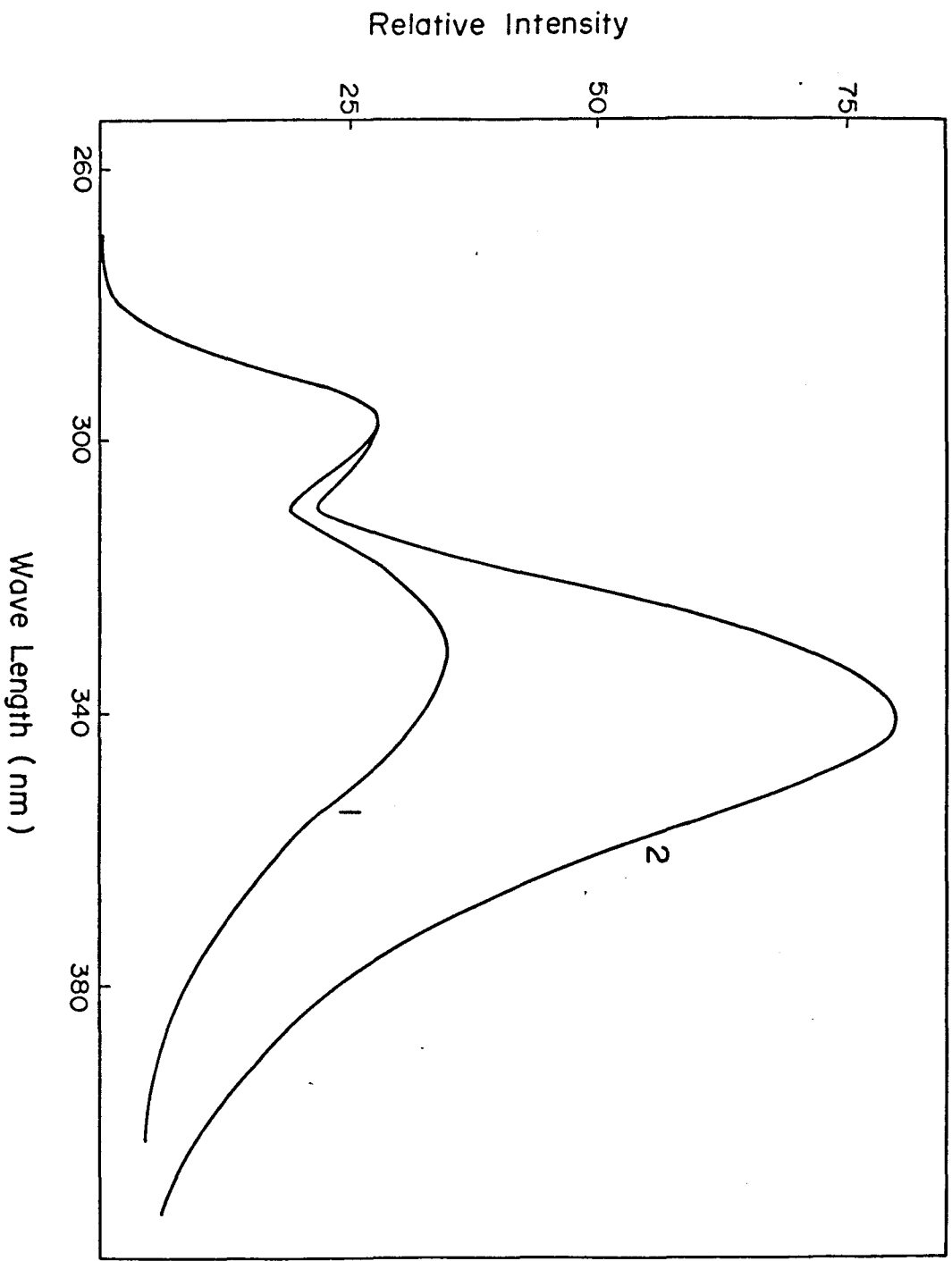
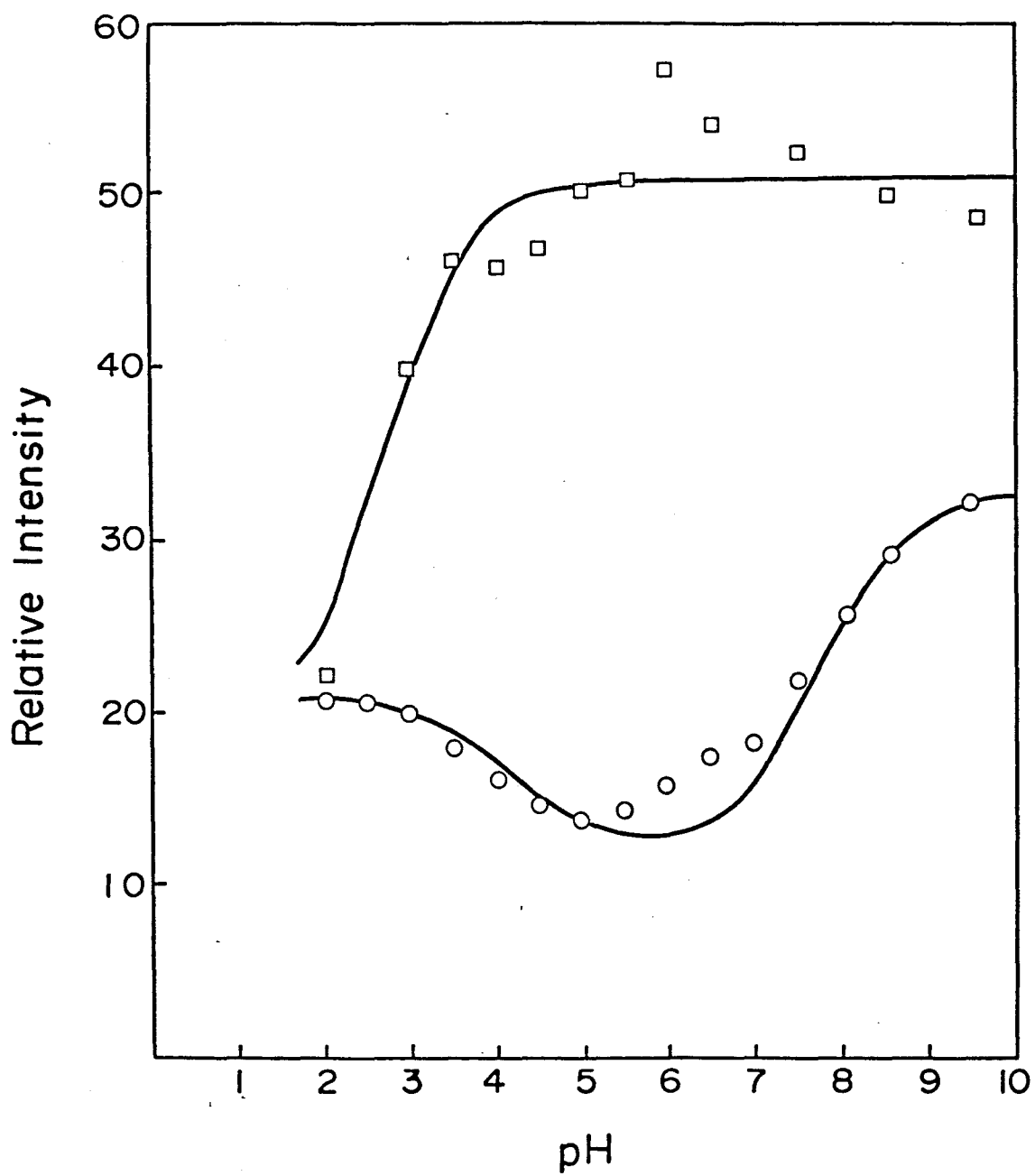


Figure 5. pH dependency of fluorescence emission of papain at 344nm in the presence and absence of N-acetyl-L-phenylalanyl-glycinal. Buffers used at each pH are described in experimental section. The excitation wavelength was 288nm. The concentration of papain and dithiothreitol were 2.07×10^{-7} M and 3.6×10^{-3} M respectively. No aldehyde, (○); 1.3×10^{-4} M aldehyde. (□)



curve) was calculated for $pK_{a1}=4.0$ and $pK_{a2}=7.8$. Inspection of the pH dependency data for the fluorescence of the complex, upper curve, indicates that the binding of aldehyde enhances fluorescence emission of papain at 344nm at all pH values between 3.0 and 9.5. Furthermore, the group with the pK_a of 7.8 in the free enzyme does not exert a significant effect on the fluorescence of the complex. The line in the upper curve was calculated for a single ionizable group in the papain aldehyde complex with a pK_a of 2.9. However, the decline in the apparent fluorescence of the complex below pH 3.0 is difficult to interpret since papain is reported to be unstable at low pH(2). The smaller variations in the fluorescence of the complex above pH 4.0 are within the limits of experimental variability encountered in measuring the fluorescence of the complex.

Measurement of the Rate of Dissociation of the Papain-N-Acetyl-L-Phenylalanyl glycinal Complex

Because of the high affinity of papain for N-acetyl-L-phenylalanyl glycinal, it is not practical to study the dissociation of the enzyme aldehyde complex by simple dilution. The dissociation of the complex can be effected at measurable enzyme levels by adding reagents which can react with and thereby trap either the free enzyme or the free aldehyde. In the presence of reagents which trap the enzyme, and under conditions of $K_s > I > EI$, the following equation described the apparent first order rate constant for the disappearance of the enzyme aldehyde complex (see section on derivations).

$$1/k_{\text{obs}} = \frac{(k_2/K_s) I_o}{k_{-2} k_T (T)} + 1/k_{-2} \quad (23)$$

In this equation, k_2/K_s is the second order rate constant for the reaction of free enzyme with aldehyde, k_T is the second order rate constant for the reaction of free enzyme with the trapping agent, sodium tetrathionate, and k_{-2} is the first order rate constant for the dissociation of the enzyme aldehyde complex. According to equation (23), k_{obs} should increase with increasing concentrations of sodium tetrathionate and should decrease with increasing initial concentrations of free aldehyde. Furthermore, plots of $1/k_{\text{obs}}$ vs $1/(T)$ at different initial aldehyde concentrations should yield a series of straight lines which intersect each other on the ordinate axis at $1/k_{\text{obs}} = 1/k_{-2}$.

Figure 6 shows first order plots of the decrease in the fluorescence of solutions containing Mercuri-Sepharose purified papain, dithiothreitol and N-acetyl-L-phenylalanyl-glycinal after an excess of Sodium tetrathionate ($T \gg RSH$) was added. As can be seen, the first order rate constant, k_{obs} , increases with increasing tetrathionate. Figure 7 shows plots of $1/k_{\text{obs}}$ vs $1/T$ at different initial aldehyde concentrations. As predicted by equation 23, the slope of the line increases with increasing aldehyde concentration. Furthermore, at each initial aldehyde concentration the k_{obs} approaches the same limiting value, k_{-2} , at infinite tetrathionate concentration. From equation (22) it can be seen that the slope of each line in Figure 7 is equal to $(\frac{k_2}{K_s}) (I)_{\text{av}}/k_{-2}k_T$, where $(I)_{\text{av}}$ is the free aldehyde concentration at 50% dissociation of the complex.

Figure 6. Determination of the pseudo first order rate constants for the dissociation of the N-acetyl-L-phenylalanyl glycinal-papain complex in the presence of varying concentrations of tetrathionate. The buffer was 0.05M acetate, 5×10^{-4} M EDTA and 0.2M NaCl, pH 4.5. The concentration of enzyme was 1.93×10^{-7} M, concentration of aldehyde was 2.35×10^{-7} M. The concentration of tetrathionate for curve (1) 1.08×10^{-4} M, curve (2) 4.8×10^{-4} M, curve (3) 4.55×10^{-3} M, and curve (4) 8.95×10^{-3} M. The temperature was $25 \pm .5^{\circ}\text{C}$. The reaction was followed by monitoring the decrease in fluorescence of the solutions at 344nm when excited at 288nm.

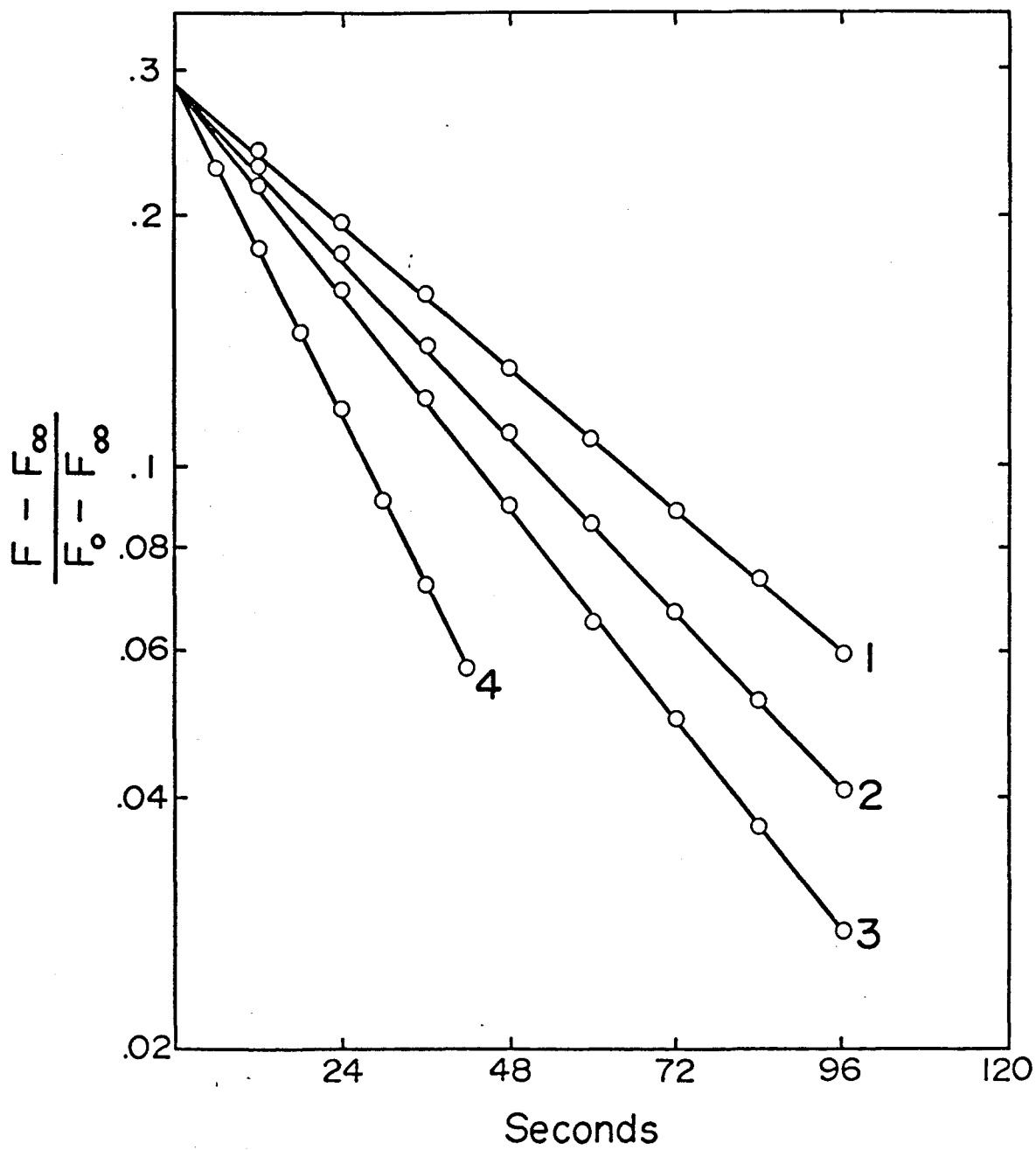
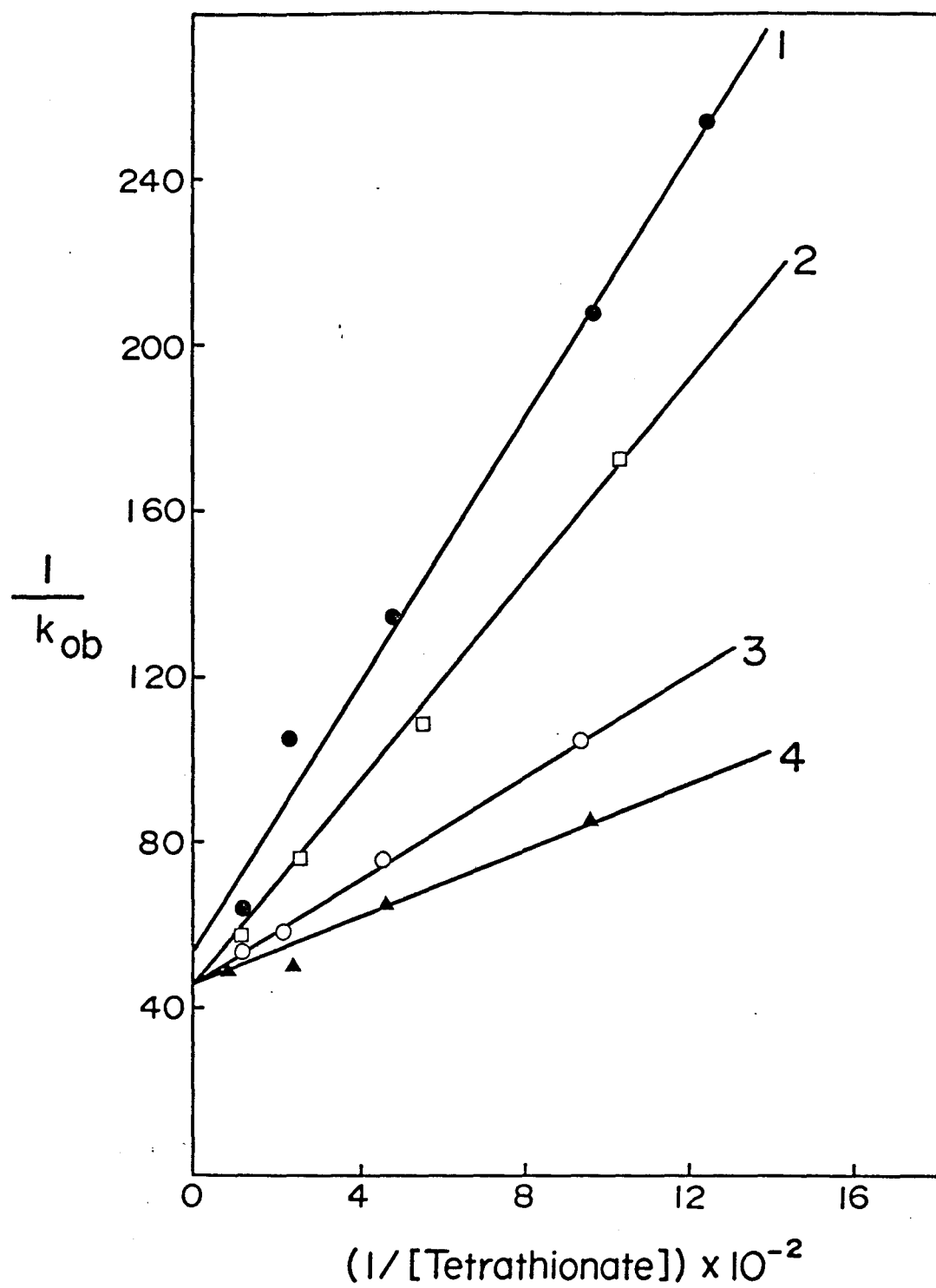


Figure 7. Dissociation of N-acetyl-L-phenylalanyl glycinal papain complex at pH 5.0 and $25 \pm .5^{\circ}\text{C}$, in the presence of varying amounts of aldehyde. The reciprocal of the first order rate constants obtained by the method illustrated in Figure 6 are here plotted as a function of the reciprocal of the tetrathionate concentrations. The intercept yields a value for k_{-2} . The buffer in this experiment was 0.05M acetate, 0.2M NaCl, $5 \times 10^{-4}\text{M}$ EDTA. Curve (1), $E_o = 2 \times 10^{-7}\text{M}$, $I_o = 7.99 \times 10^{-7}\text{M}$; curve (2), $E_o = 2 \times 10^{-7}\text{M}$, $I_o = 6.1 \times 10^{-7}\text{M}$; curve (3), $E_o = 1.7 \times 10^{-7}\text{M}$, $I_o = 4.66 \times 10^{-7}\text{M}$; curve (4), $E_o = 1.7 \times 10^{-7}\text{M}$, $I_o = 2.4 \times 10^{-7}\text{M}$.



From the slope it is therefore possible to calculate the ration $(k_2/K_s)/k_{-2}k_T$.

Figure 8 and Table III show the pH dependency of the first order rate constant, k_{-2} , for the dissociation of the enzyme-aldehyde complex. It can be seen that k_{-2} is pH independent between pH 5.0 and 8.5. Between pH 5.0 and 3.0, k_{-2} shows a small increase with decreasing pH. The data can be fit by a model which assumes the existence of two inter-convertible forms of the enzyme aldehyde complex, each characterized by a particular value for k_{-2} . This model leads to the following equation.

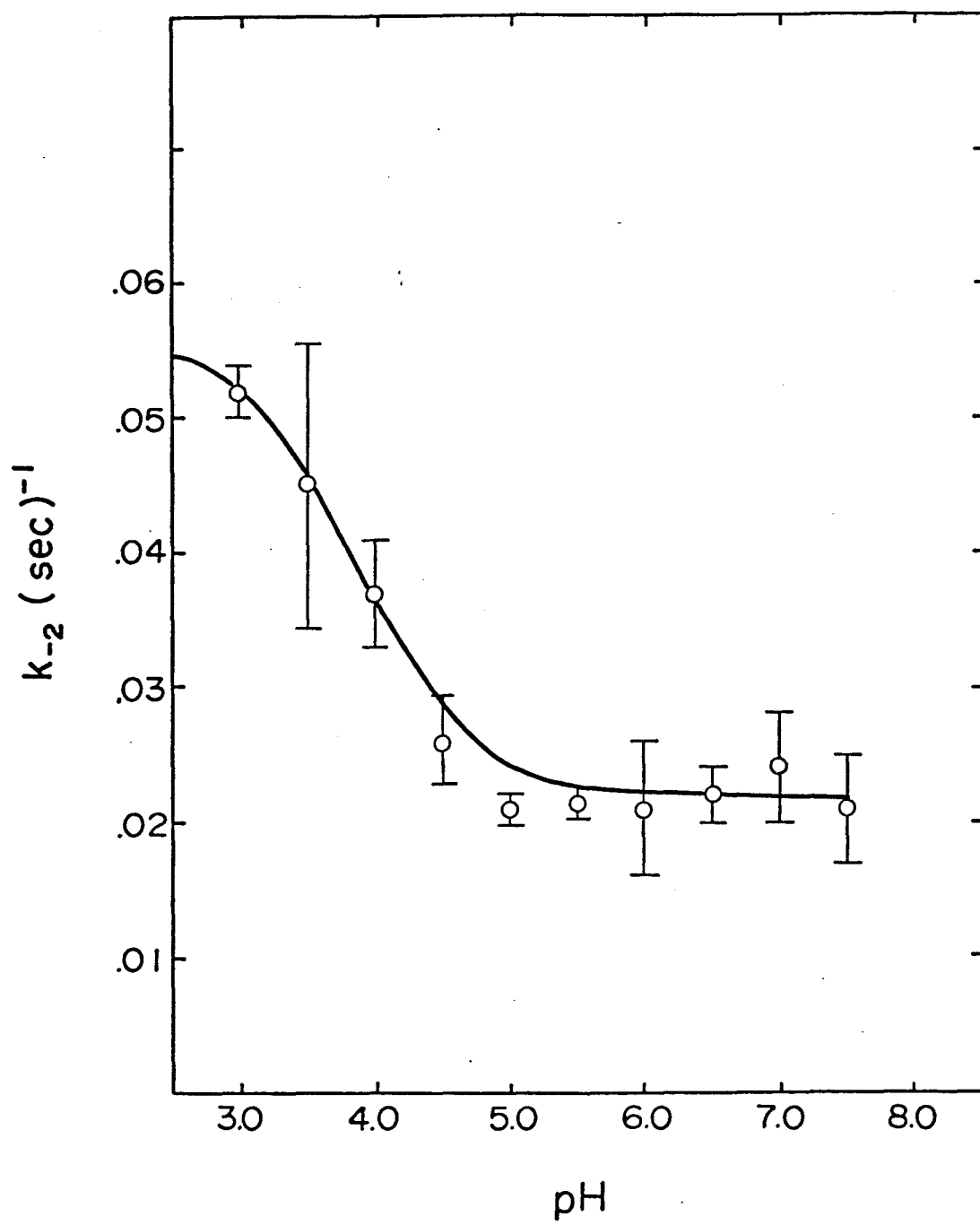
$$(k_{-2})_{\text{obs}} = \frac{k_{-2}^H (H) + k_{-2}^o K_a}{(H) + K_a} \quad (26)$$

In this equation, k_{-2}^H is the limiting value of k_{-2} for the form of the complex at low pH, k_{-2}^o is the limiting value for the form of the complex at higher pH and K_a is the acid dissociation constant of the group in the complex which controls the interconversion of the two forms of the enzyme.

The open circles in Figure 8 are the experimental points and the solid line was calculated using values for k_{-2}^H , k_{-2}^o , and pK_a of .056, .022, and 3.9 respectively. Alternatively, the data can also be made to fit an equation of the form $(k_{-2})_{\text{obs}} = k_{-2}^o + k_{-2}^H (H)$ (26a)

In equation 26a, k_{-2}^o is the rate constant for an uncatalyzed decomposition of the hemitheoacetal and k_{-2}^H is the rate constant for the decomposition of the hemitheoacetal via a pathway involving protonation of the sulfur prior to the cleavage of the carbon-sulfur bond (specific and catalysis).

Figure 8. The pH dependency for the rate constant of dissociation of N-acetyl-L-phenylalanyl-glycinal at $25 \pm .5^{\circ}\text{C}$. Buffers used are described in experimental section. k_{-2} at each pH was obtained with the aid of equation (23) as described in Figure 7. In each case, concentration of N-acetyl-L-phenylalanyl-glycinal was $2.4 \times 10^{-7}\text{M}$.



Second Order Rate Constants for the Association of Papain with N-Acetyl-L-Phenylalanyl glycinal

Equation (24) describes the relationship between the overall association constant for the binding of an aldehyde to papain and the rate and equilibrium constants for the individual steps in this reaction.

$$1/K_I = (k_2 + k_{-2})/k_{-2} K_S \quad (24)$$

For N-acetyl-L-phenylalanyl glycinal at pH 6.5, it has been shown that, k_2 is more than 10^3 times greater than k_{-2} (24). Therefore, $1/K_I \approx k_2/K_S \times 1/k_{-2}$.

With this relationship, the second order rate constant for the reaction of papain with N-acetyl-L-phenylalanyl glycinal can be calculated. These values are summarized in Table III and plotted as a function of pH in Figure 9. The circles are the experimental points and the solid line was calculated assuming a group on the free enzyme must be in an unprotonated form in order for reaction with aldehyde to occur. The pK_a of this group is 3.9 and the limiting value of k_2/K_S is $2.75 \times 10^6 M^{-1} sec^{-1}$. Table IV is a summary of the limiting values of $1/K_I$, k_{-2} and k_2/K_S together with the apparent pK_a values of groups on the enzyme which control those constants.

Figure 9. The pH dependency for the association of N-acetyl-L-phenylalanyl glycinal with papain at 25°C. The buffers used in this experiment are described in methods. The association rate constant, k_2/K_s , was calculated at each pH using the experimentally determined values of K_I and k_{-2} as described in the results.

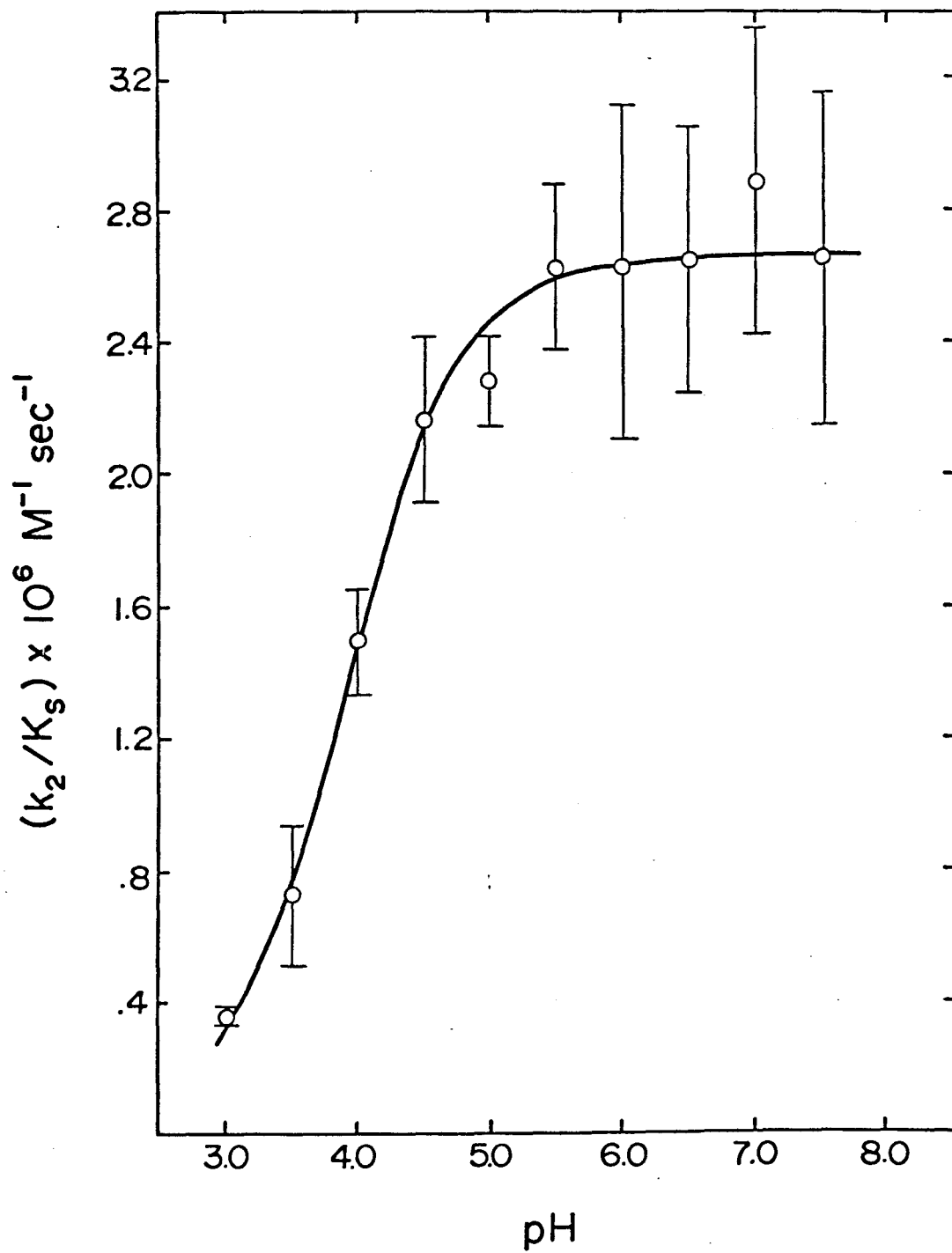


TABLE III

pH dependency of the individual kinetic constants for the association of N-acetyl-L-phenylalanyl glycinal with papain

pH	$(1/K_I) \times 10^{-8} M^{-1}$	$k_{-2} \text{ sec}^{-1}$	$(k_2/K_S)^b \times 10^{-6} M^{-1} \text{ sec}^{-1}$
3.0	$(0.065)^a$	$0.052 \pm .003$	(0.28)
3.5	$0.17 \pm .01$	$0.045 \pm .013$	$0.77 \pm .21$
4.0	$0.39 \pm .03$	$0.037 \pm .004$	$1.44 \pm .19$
4.5	$0.83 \pm .03$	$0.027 \pm .003$	$2.23 \pm .27$
5.0	$1.08 \pm .08$	$0.022 \pm .001$	$2.32 \pm .15$
5.5	$1.23 \pm .10$	$0.022 \pm .001$	$2.74 \pm .25$
6.0	$1.26 \pm .04$	$0.022 \pm .005$	$2.74 \pm .62$
6.5	$1.21 \pm .14$	$0.023 \pm .002$	$2.78 \pm .39$
7.0	$1.21 \pm .15$	$0.023 \pm .004$	$2.78 \pm .60$
7.5	$1.25 \pm .05$	$0.022 \pm .004$	$2.69 \pm .52$
8.0	$1.25 \pm .10$	$0.022 \pm .004$	$2.75 \pm .56$

a Calculated according to equation 25 for $pK_a = 4.25$

b Calculated according to equation (27)

TABLE IV

Limiting Values for the Kinetic Parameters of the Association of
N-acetyl-L-phenylalanyl glycinal with Papain

Constant	Limiting Value	$(pK_a)_{app}$
$1/K_I$	$1.25 \times 10^8 M^{-1}$	4.25
k_{-2}	.023, .056 sec^{-1}	3.9
k_2/K_s	$2.75 \times 10^6 M^{-1} sec^{-1}$	3.9

CHAPTER VI

DISCUSSION

Introduction

Substantial evidence exists indicating that the pathway of papain catalyzed hydrolysis reactions include the formation and decomposition of a thiolester acyl-enzyme intermediate (10, 11, 12). Mechanistically both acylation and deacylation have been shown to be substitution reactions at the carbonyl carbon of the substrate, with the cysteine sulfhydryl and water acting as nucleophiles. With few exceptions, non-enzymatic acyl substitution reactions occur through the formation of an acyl-nucleophile adduct in which the carbonyl carbon assumes a tetrahedral configuration (32, 33). Although tetrahedral intermediates have also been postulated on the pathway of enzymatic acyl transfer reactions (34, 35), the alternative suggestion has been advanced that such reactions could occur directly via a single transition state (36). Studies aimed at resolving the question for papain have provided evidence suggestive of additional intermediates between Michaelis complex and the acyl enzyme. In most cases, the identity of these intermediates have not been established.

Recently, Angelides and Fink (37) have obtained direct spectrophotometric evidence for a tetrahedral intermediate in low temperature studies of papain catalyzed hydrolysis of N-carbobenzoxy-L-lysine p-nitroanilide. On mixing this substrate with papain, they reported trapping an intermediate at -3°C in 60% aqueous DMSO whose absorption spectrum resembled

that predicted for the tetrahedral intermediate. They also detected the presence of an additional intermediate, preceding the tetrahedral intermediate on the reaction pathway, which they identified as an "activated" Michelis Complex (38). In contrast, low temperature studies with N-alpha-carbobenzoxy-L-lysine p-nitrophenylester revealed only the 'activated' Michelis Complex (39).

Other less direct evidence for a tetrahedral intermediate in papain catalysis comes from studies of structure - reactivity relationships in substrate hydrolysis. Using substituted phenyl esters and anilides of hippuric acid, Lowe and Yuthavong (40) obtained values which they interpreted as indicating that formation of a tetrahedral intermediate is rate determining for aryl esters and breakdown is rate determining for anilides. O'Leary et al. (41) determined a $^{14}\text{N}/^{15}\text{N}$ kinetic isotope effect in N-benzoyl-L-arginine amide hydrolysis and concluded that a tetrahedral intermediate is formed whose breakdown is rate determining for overall catalysis at neutral pH with this substrate. Other suggestive evidence is derived from the X-ray structure of a papain inhibitor complex. In this structure, a notable feature is the "oxyanion hole" whereby the oxide moiety of a presumptive anionic tetrahedral intermediate could be stabilized by hydrogen bonding to the NH peptide backbone of cysteine 25 and the $\epsilon\text{-NH}_2$ of glutamine 19(42).

Studies on the binding of aldehyde analogs of acylamino acids and dipeptides to papain, suggest that they react with the enzyme in two steps (24). From cross-saturation nuclear magnetic resonance studies, step 1 can probably be identified as the formation of a noncovalent complex

(Michelis complex) between the unhydrated aldehyde and papain, and step 2 as the formation of a covalent, tetrahedral hemithioacetal (23). Further evidence for hemithioacetal formation is derived from comparison of binding constants of aldehyde derivatives with those of related amides, nitriles, alcohols and methylketones (22), from observation of a measurable secondary isotope effect in aldehyde binding (28) and from studies of aldehyde binding to $-S-CH_3$ Cysteine modified papain (24). It is suggested that the unusual stability of these hemithioacetals in papain is due to their resemblance to the proposed tetrahedral transition state (and tetrahedral intermediate) in substrate hydrolysis (22). It is, therefore, proposed that the binding of aldehydes to papain are model reactions for tetrahedral intermediate formation during substrate catalysis (44).

Aspects of the mechanism of papain which are still unclear are the identity of the groups on the enzyme responsible for the observed pH dependence of catalysis, their protonic form within the active enzyme, and their precise role in catalysis. These mechanistic uncertainties reflect the presence within the papain active site of three ionizable amino acid residues, cysteine 25, histidine 159, and aspartate 158, in sufficient proximity to mutually interact with substrate (7). This leads to serious ambiguities in assigning a mechanism for papain based on the pH dependency of steady state kinetic parameters for substrate hydrolysis. Thus, one can write at least two plausible mechanisms for papain which are based on the interaction of histidine 159 and cysteine 25 with substrate and which are consistent with current experimental data. These are illustrated in the introduction. Still other mechanisms are possible for papain assuming participation of aspartate 158 in catalysis (43).

To distinguish between the various possible mechanisms for papain, it is necessary to determine unambiguously the pK and, thereby, the protonic form of cysteine 25 in active papain. One approach has been to study the pH dependency of the change in the proton content in papain during the reversible interconversion to its S-thiomethane derivative (21). From this data two macroscopic ionization constants were calculated for the papain active site. It is then necessary to measure independently one of four microscopic ionization constants in native papain in order to calculate the remaining three from the macroscopic titration constants. An independent measurement of one of these four microscopic dissociation constants can not be made easily. Instead, a value for one of them was assigned from fluorescence titration data with S-methylthio papain. On this basis it was concluded that histidine 159 and cysteine 25 existed as a thiolate-imidazoleum ion pair in the native enzyme. This conclusion, however, requires two unconfirmed assumptions; the pH dependency of the fluorescence of S-methylthio papain is attributable solely to the ionization of histidine 159 and the dissociation constant for histidine 159 in S-methylthio-papain is identical with the microscopic dissociation constant for histidine 159 in the fully protonated state of native papain. An alternative assignment of one of the microscopic ionization constants leads to the opposite conclusion (43).

Another approach to this problem is to measure the pK_a of cysteine 25 in an elementary step on the catalytic pathway in which it is a participant (39), preferably, the step in which the tetrahedral intermediate is formed. Efforts to isolate the individual steps in papain catalysis by low temperature stabilization of reaction intermediates have provided

considerable information about pH dependent transitions within the free enzyme and the Michelis complex and has allowed detection of a tetrahedral intermediate (6, 7). Although the results of this work can be interpreted within the framework of a mechanism for papain involving the participation of a thiolate-imidazoleum ion pair, this mechanism was not unequivocally established.

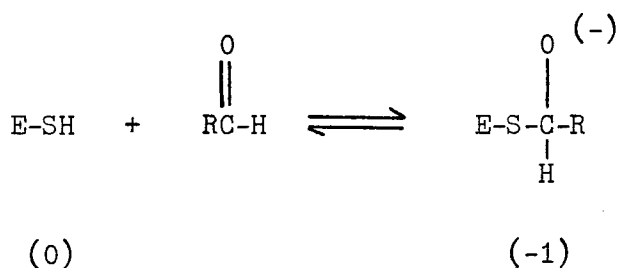
In this study we have investigated the effect of pH on the binding of an aldehyde analog of a substrate since this reaction is a putative model for the formation and retrogression of a tetrahedral intermediate in papain catalysis. In the remainder of this chapter it will be shown that the results of this work provide strong and relatively unambiguous evidence for a mechanism for aldehyde binding involving the unassisted attack of the thiolate anion of cysteine 25 on carbonyl carbon to produce a negatively charged tetrahedral adduct. This adduct can then retrogress to the Michelis complex by an unassisted expulsion of the thiolate anion. Extension of this result, to the reactions with substrates, provides strong support for a mechanism for papain involving a thiolate-imidazoleum ion pair in catalysis.

pH Dependency of Equilibrium Association Constant for N-acetyl-L-Phenylalanyl glycinal with Papain

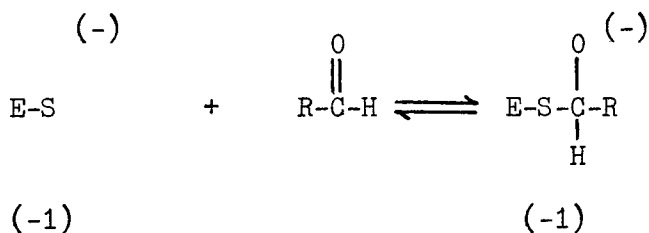
As shown in Figure 3, pH dependency of $1/K_I$ is a simple sigmoid curve characteristic of the ionization of a single group with a pK_a of 4.25. When the log of dissociation constant is plotted as a function of pH, one obtains two straight lines one with a slope of zero and one with a slope of +1 connected by a short segment. According to the rules

developed by Dixon (45), this logarithmic plot may be interpreted to mean that a single substituent which can ionize in the free enzyme is no longer able to ionize in the enzyme inhibitor complex. Second, that below pH 4.0 the difference in charge between the free enzyme and the enzyme inhibitor complex is +1. Above pH 5.0 the difference in charge between the free enzyme and the EI complex is zero. The simplest scheme consistent with these results is as follows:

Below pH 4.0



Above pH 5.0



In this scheme the nucleophile is identified as the group on the free enzyme whose ionization is prevented on forming the enzyme inhibitor complex. This scheme requires that the tetrahedral thiohemiacetal adduct is negatively charged in the complex. Presumably the Oxyanion can not be protonated because it occupies the oxyanion hole in the enzyme active site. The pH dependency of $1/K_I$ thus provides strong evidence that active site

nucleophile is already unprotonated in the active form of papain above pH 5.0. It also provides support for the presence of an oxyanion hole in the papain active site as proposed from the X-ray structure of papain (42). Furthermore, the simple pH dependency observed argues that the ionization of other groups in the pH range between 3.5 and 8.0 at the active site of papain are not significantly perturbed on forming the complex (45).

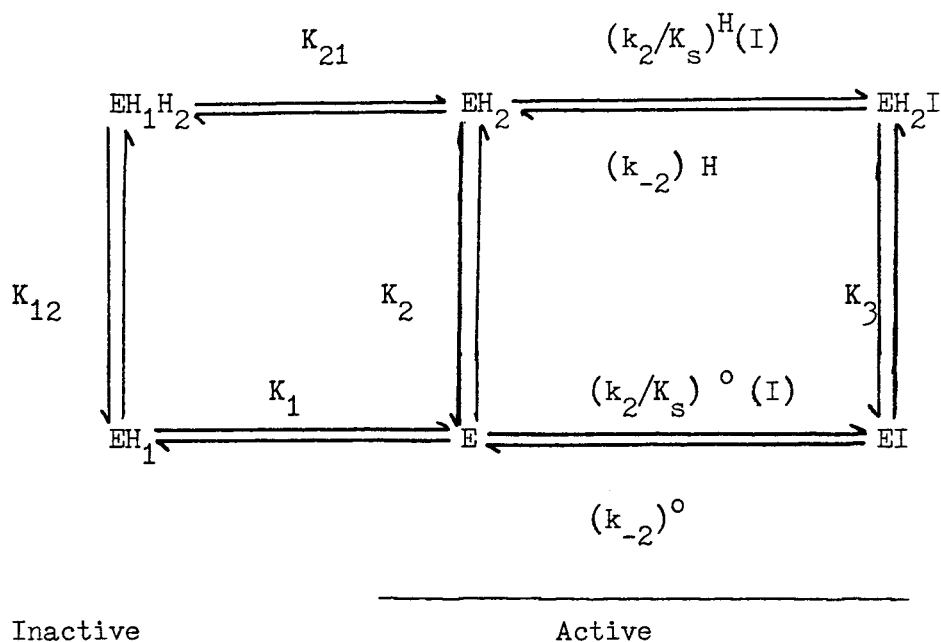
pH Dependency of the Dissociation Rate Constant, k_{-2}

Since the active site nucleophile is already ionized in the active form of papain, there is no need for a second group on the enzyme to act as a general base during the formation of the complex. Since the dissociation of the enzyme-aldehyde complex the microscopic reverse of association reaction it follows that there is no need for a general acid to facilitate the decomposition of the complex. This leads to the prediction that k_{-2} should be essentially pH independent. This was confirmed as shown in Figure 8. The small perturbation in k_{-2} observed below pH 4.0 only increases its value by a factor of 2 to 3 indicating that the group being protonated is not essential for dissociation to occur.

pH Dependency of Second Order Rate Constant for the Association of Aldehyde to Papain

From K_I and k_{-2} it is possible to calculate k_2/K_S as discussed in results. Since k_2/K_S represents a second order rate constant for the reaction of free enzyme with aldehyde, its pH dependency can be used to determine the dissociation constant of the active site nucleophile. The pK_a of active site nucleophile is about 3.9 from Figure 9.

Ionization of Amino Acid Residues at the Papain Active Site



The pH dependencies of the kinetic constants for the association of N-acetyl-L-phenylalanyl glycinal with papain can be described by the scheme shown above. According to this scheme, the active site contains two ionizable groups with similar pK_a values such that $pK_{a1} \approx pK_{a2} \approx pK_{a3}$. Several investigators have obtained evidence that the activity of papain is affected by the ionization of two acidic residues at the enzyme active site (21). Group 1 is identified as the nucleophile (Cysteine 25) and its protonation leads to enzyme inactivation. The identity of group 2 is unknown. However, one possible candidate is Aspartate 158. Protonation of this group alters but does not abolish enzyme activity. This scheme leads to the following equations for the pH dependency of the kinetic constants for aldehyde association.

$$(k_2/K_s)_{\text{obs}} = \frac{(k_2/K_s)^o + (k_2/K_s)^H \times \frac{(H)}{K_{a1}}}{\left(1 + \frac{(H)}{K_{a1}}\right) \left(1 + \frac{(H)}{K_{a2}}\right)} \quad (28)$$

When $(k_2/K_s)_{\text{obs}} = (k_2/K_s)^H$, and $pK_1 = pK_2$, this simplifies to equation 25

$$(k_2/K_s)_{\text{obs}} = \frac{(k_2/K_s)_{\text{lim}}}{1 + \frac{(H)}{K_a}} \quad (25)$$

Similarly, according to this scheme, k_{-2} is given by equation 26

$$(k_{-2})_{\text{obs}} = \frac{k_{-2}^H (H) + k_{-2}^o K_a}{(H) + K_a} \quad (26)$$

Rearranging equation 26 we can write

$$(k_{-2})_{\text{obs}} = \frac{k_{-2}^o \left(1 + \frac{k_{-2}^H}{k_{-2}^o} \frac{(H)}{K_{a1}}\right)}{1 + \frac{H}{K_a}} \quad (29)$$

We can define a new apparent ionization constant

$$K_{\text{app}} = K_a \frac{k_{-2}^o}{k_{-2}^H}$$

$$(k_{-2})_{\text{obs}} = \frac{(k_{-2}^0) \left(1 + \frac{H}{K_{\text{app}}}\right)}{1 + \frac{H}{K_a}} \quad (30)$$

Since $1/K_I = (k_2/K_s) / k_{-2}$ we can write

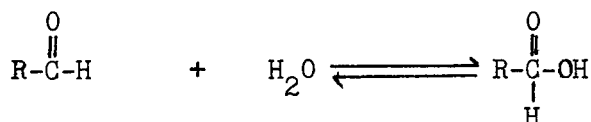
$$(1/K_I) = \frac{(k_2/K_s)_{\text{lim}}}{\left(1 + \frac{H}{K_a}\right)} \cdot \frac{\left(1 + \frac{H}{K_a}\right)}{k_{-2}^0 \left(1 + \frac{H}{K_{\text{app}}}\right)} \quad (31)$$

$$(1/K_I)_{\text{obs}} = \frac{(1/K_I)_{\text{lim}}}{1 + \frac{H}{K_{\text{app}}}} \quad (32)$$

This means that the K_a governing the pH dependency of $1/K_I$ should be equal to the product of the K_a of the active site residue and the ratio of k_{-2}^H/k_{-2}^0 . Since $k_{-2}^H/k_{-2}^0 = 2.5$, pK_{aapp} should be equal to $pK_1 + \log 2.5$. For $pK_1 = 3.9$ (see results) $pK_{\text{aapp}} = 4.3$ in good agreement with the experimental value of 4.25.

Effects of Hydration of the Aldehyde on the Kinetic Constants

Thus far, kinetic constants for aldehyde binding to papain were calculated on the assumption that all the aldehyde in solution was free to react with the enzyme. It has been shown that N-acetyl-L-phenylalanyl-glycinal becomes extensively hydrated in water as shown below.



The value for the hydration constant $K_h = \frac{\text{Hydrated aldehyde}}{\text{Free Aldehyde}}$

is 7.9 (44). Since it is the unhydrated aldehyde which reacts with papain (44, 23), the kinetic constants must be corrected for this hydration reaction. The effect of this correction is to decrease K_I by a factor of 8.9 and increase k_2/K_s by this same factor. Hydration has no effect on k_{-2} . The corrected values for K_I and k_2/K_s become $9 \times 10^{-10} \text{M}^{-1}$ and $2.55 \times 10^{-7} \text{M}^{-1} \text{sec}^{-1}$ respectively.

Identity of the Active Site Nucleophile

Although the pH dependency data discussed so far provides strong evidence that an unprotonated nucleophile at the papain active site reacts with aldehyde to form a negatively charged covalent tetrahedral adduct, the data do not identify the nucleophile. In this work we have assumed that the nucleophile is the thiolate anion of cysteine 25 since this residue functions as a nucleophile in catalysis. Support for the assumption comes from the structural similarity of the aldehyde to carboxylic acid substrates. Additional support comes from the magnitude of k_2/K_s for the reaction of the aldehyde with papain. At pH 6.0 the value of k_2/K_s corrected for hydration of aldehyde is about $2.6 \times 10^7 \text{M}^{-1} \text{sec}^{-1}$. This value is equal or greater in magnitude than the second order rate constants for acylation of papain by its most specific substrates. This leads to the conclusion that the binding of aldehyde can not be considered to represent a nonspecific covalent modification of some other active site residue. The large magnitude of k_2/K_s for the aldehyde and its similarity to corresponding values for substrate catalysis suggest that aldehyde is reacting with papain by a mechanism similar to that of the substrate.

In conclusion, the data presented in this work is evidence that the sulfhydryl group of cysteine 25 is already unprotonated in papain above pH 5.0. Thereby lending support to mechanisms for papain catalysis involving a thiolate imidazoleum ion pair at the enzyme active site.

REFERENCES

- (1) Kimmel, J. R. and Smith, E. (1957) *Advances in Enzymology* 19, 267
- (2) Glazer, A. N. and Smith, E. (1971) *Enzymes* 3, 501
- (3) Kimmel, J. R. and Smith, E. L. (1954) *J. Biol. Chem.* 207, 515
- (4) Glazer, A. N. and Smith, E. (1965) *J. Biol. Chem.* 240, 201
- (5) Blumberg, S., Schechter, I., Berger, A. (1970) *European Journal of Biochemistry* 15, 97
- (6) Drenth, J., Jansonius, R., Koekoek, R., Swen, H. M., Wolthers, B. G. (1968) *Nature* 218, 929
- (7) Drenth, J., Jansonius, J., Koekoek, R., and Wolthers, B. G. (1971) *Enzymes*, 3, 484
- (8) Blow, L. and Steitz, R. (1970) *Ann. Rev. Biochem.* 39, 89
- (9) Lowe, G. (1970) *Phil. Trans. Roy. Soc. Lond.* B257, 237
- (10) Bender, M. L., Begue-Canton, M. L., Blakely, R. L. Brubacher, L. Feder, J. Gunter, C. L., Kézdy, F. J. (1966) *J. Amer. Chem. Soc.* 88, 5890
- (11) Bender, M. L. and Brubacher, L. J. (1964) *J. Amer. Chem. Soc.* 86, 5333
- (12) Lowe, G. and Williams, A. (1965) *Biochem. J.* 96, 189
- (13) Loffler, H. and Schnider, A. (1974) *FEBS Letters* 45, 79
- (14) Husain, S. and Lowe, G. (1968) 108, 855
- (15) Husain, S. and Lowe, G. (1968) 108, 861
- (16) Shinitzky, M. and Goldman, R. (1967) *European Journal of Biochemistry*, 3, 139
- (17) Whitaker, J. and Bender, M. (1965) *J. Amer. Chem. Soc.* 87, 2728
- (18) Weinryb, I. and Steiner, R. F. (1970) *Biochemistry* 9, 135
- (19) Murachi, T., Tsudzuki, T., Okumura, K. (1975) *Biochemistry* 14, 249

- (20) Polgar, L. (1973) European Journal of Biochemistry 33, 104
- (21) Lewis, S. D., Johnson, F. A., Shafer, J. A. (1976) Biochemistry 15, 5009
- (22) Westerik, J. and Wolfenden, R. (1972) J. Biol. Chem. 247, 8195
- (23) Bendall, M. R., Cartwright, I. L., Clark, P. J., Lowe, G., Nurse, D. (1977) Eur. J. Biochem. 79, 201-209
- (24) Mattis, P., Hemes, J., Fruton, J. (1977) J. Biol. Chem. 252, 6776
- (25) Bender, M. and Brubacher, L. J. (1966) J. Amer. Chem. Soc. 88, 5880
- (26) Husain, S. and Lowe, G. (1970) Biochem. J. 116, 689
- (27) Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1967) J. Amer. Chem. Soc. 89, 5012
- (28) Lewis, C. and Wolfenden, R. (1977) Biochemistry 16, 4886
- (29) Barret, A. J. (1973) Biochem. J. 131, 811
- (30) Axen, R. and Ernback, S. (1971) Eur. J. Biochem. 18, 351
- (31) Sluyterman, L. A. E. and Wijdenes, J. (1970) Biochem. Biophys. Acta. 200, 593
- (32) Bender, M. L. (1960) Chem. Reviews 60, 53
- (33) Fedor, L. R. and Bruce, T. C. (1964) J. Amer. Chem. Soc. 86, 5697
- (34) Frankfater, A. and Kézdy, F. J. (1971) J. Amer. Chem. Soc. 93, 4039
- (35) Hunkapillar, M. W., Forgac, M. D., Richards, J. H. (1976) Biochemistry 15, 5581
- (36) Fastrez, J., and Fersht, A. R. (1973) Biochemistry 12, 1067
- (37) Angelides, K. and Fink, A. (1979) Biochemistry 18, 2355
- (38) Angelides, K. and Fink, A. (1979) Biochemistry 18, 2363
- (39) Angelides, K. and Fink, A. (1978) Biochemistry 17, 2659

- (40) Lowe, G and Yuthavong, Y. (1971) Biochem. J. 124, 117
- (41) O'Leary, M. H. and Urber, M. and Young, A. P. (1974) Biochemistry 13, 2077
- (42) Drenth, J., Kalk, K. H. and Swen H. M. (1976) Biochemistry 15, 3731
- (43) Zannis, V. I. and Kirsch, J. F. (1978) Biochemistry, 17, 2669
- (44) Lewis, C. and Wolfenden, R. (1977) Biochemistry, 16, 4890
- (45) Dixon, M. (1953) Biochem. J. 55, 161
- (46) Perlstein, S. H. and Kézdy, F. J. (1973) Supra Mol. Struct 1, 249

APPROVAL SHEET

The thesis submitted by Thresiamma Kuppy has been read and approved by the following committee:

Allen Frankfater Ph.D., Director
Associate Professor, Biochemistry and Biophysics
Loyola, Stritch School of Medicine

Richard M. Schultz, Ph.D.
Associate Professor, Biochemistry and Biophysics
Loyola, Stritch School of Medicine

Ferenc J. Kézdy, Ph.D.
Professor, Biochemistry
The University of Chicago

The final copies have been examined by the Director of thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

December 9, 1980
Date

Allen Frankfater
Director's Signature